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THE EFFECTS OF PRENATAL PCBs ON FEMALE REPRODUCTION: DEVELOPMENT, BEHAVIOR, AND GENE EXPRESSION

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Dedication

I dedicate this dissertation to my grandfather, Edward J. Stranke, who wanted very much to be here today. In addition, this work is dedicated to my boyfriend Adam Brown, my supportive parents Susan and Michael Steinberg, and Dr. Andrea Gore, whose faith in my abilities made all of this possible.

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The University of Texas at Austin, 2007

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Polychlorinated biphenyls (PCBs) are a class of bioactive chemical once used in industrial applications, but which now contaminate the world environment. PCBs are lipophilic with few natural degadatory mechanisms, and thus they accumulate in human and animal tissues, and are passed to subsequent generations via transfer between mother and offspring. Research has shown that PCBs can interfere with brain and sexual organ development, and adult sexual behaviors and reproduction. However, previous studies produced contradictory results based on the dose and method of administration, species, and the age at exposure. The research detailed in this thesis elucidates the effects of prenatal exposure to low levels of a commercial mixture of PCBs, Aroclor (A) 1221, on female reproductive function.

The studies undertaken in this dissertation focus on three areas relevant to understanding long-term effects of PCBs on reproductive

physiology in female rats: (1) developmental effects in two generations, (2) sexual behaviors in the first generation, and (3) gene expression in the first generation. In the first research section of this dissertation, the sexual and somatic development of PCB-exposed animals is investigated in first (F1) and second (F2) generation females. Dose-dependent effects are observed in both generations, and a greater number of endpoints are significantly affected in the F2, including circulating hormone levels and uterine and ovarian weight. The second research section of the dissertation explores whether sexual behaviors in the first generation of exposed animals are altered by A1221, using a paced mating paradigm designed to elucidate female-typical behaviors. Several salient behaviors are affected by PCB exposure, including likelihood to mate, mating trial pacing, and stress-related vocalizations. The third research chapter discusses the results of a genome-wide microarray assay performed on the preoptic area of the brain. The preoptic area is a neuroendocrine control center implicated in regulation of reproductive physiology and behavior. Taken together, these results suggest that A1221 has long lasting and trans-generational effects on the development and behavior of exposed females, accompanied by altered gene expression in a neuroendocrine region of the brain. These findings have implications for female reproductive health and reproductive success in wildlife and humans.

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Nomenclature and Abbreviations

A	**Aroclor**
ACTH	**Adrenocorticotropic hormone**
AP1	**Activating protein 1**
APP	**Amyloid beta precursor protein**
aRNA	**amplified ribonucleic acid(s)**
BCA3	**breast cancer associated oncogene 3**
bp	**base pair(s)**
cDNA	**complementary deoxyribonucleic acid(s)**
CREB	**cAMP-responsive binding element**
CRH	**corticotropin releasing hormone/factor**
CRHBP	**corticotropin releasing hormone binding protein**
CRHR	**corticotropin releasing hormone receptor (1 or 2)**
DES	**diethylstilbestrol**
DMSO	**dimethyl sulfoxide**
E	**embryonic day**
ECD	**electron capture detector**
EDC	**endocrine disrupting chemical**
EGRF	**Early Growth Factor family of transcription factors**
ELAV	**embryonic-lethal abnormal vision (gene)**
Elk-1	**Ets-like gene 1**
EPA	**Environmental Protection Agency**
ER	**estrogen receptor**
EREF	**estrogen receptor family of transcription factors**
ERK8	**extracellular signal-regulated kinase 8**

Ets-1	**Avian retroviral E-Twenty-Six family of transcription factors**
FDR	**false discovery rate**
GABF	**GAGA box transcription factor family**
GO	**gene ontology**
GCOS	**GeneChip Operating Software**
GnRH	**gonadotropin releasing hormone**
HEAT	**heatshock factors family of transcription factors**
HOXF	**homeobox transcription factor family with moderate activity**
HPA	**hypothalamic-pituitary-adrenal**
HRGC	**high resolution gas chromatography**
Ihop	**Information Hyperlinked Over Proteins**
LQ	**lordosis quotient**
MM	**mismatch probe**
MPSS	**multiprotein survey system**
mRNA	**messenger ribonucleic acid**
MS	**selected ion monitoring mass spectrometer**
NKXH	**Nkx homeobox family of transcription factors**
NTP	**nucleoside triphosphate**
NRF1	**Nuclear Respiratory Family of transcription factors**
OMIM	**Online Mendelian Inheritance in Man**
P	**postnatal day**
PCB	**polychlorinated biphenyl**
PCDF	**polychlorinated dibenzofuran**
PCQ	**polychlorinated quaterphenyls**
PKC	**protein kinase C**

PM	**perfect match probe**
POA	**preoptic area (of the brain)**
RMA	**robust multichip average**
Sp1	**Stimulating Proteins 1 family of transcription factors**
TRE	**transcriptional response element**
TRHR	**thyrotropin releasing hormone receptor**
TTFF	**thyroid hormone receptor family of transcription factors**

General Introduction

General Information on PCBs

HISTORY

Polychlorinated biphenyls (PCBs) are a class of bioactive industrial chemical composed of 209 possible permutations (congeners) of chlorine substitutions on a biphenyl frame. Although small scale PCB production began in 1881 in the United States, mass commercial production in America was initiated in 1929 in response to a demand for safer insulating materials in industrial machinery. Designed for encapsulation in closed-system apparatuses such as transformers and capacitors (Carpenter, 1998), PCBs were soon also employed in such divergent applications as plastics, nautical paint, microscope oil, pesticides, copy paper, adhesives, and sealants. Ironically, the same chemical properties that render PCBs valuable for industry also contribute to their toxicity. The chemical structure of PCBs is given in Figure 1.

Figure 1: PCB Congeners and Classifications

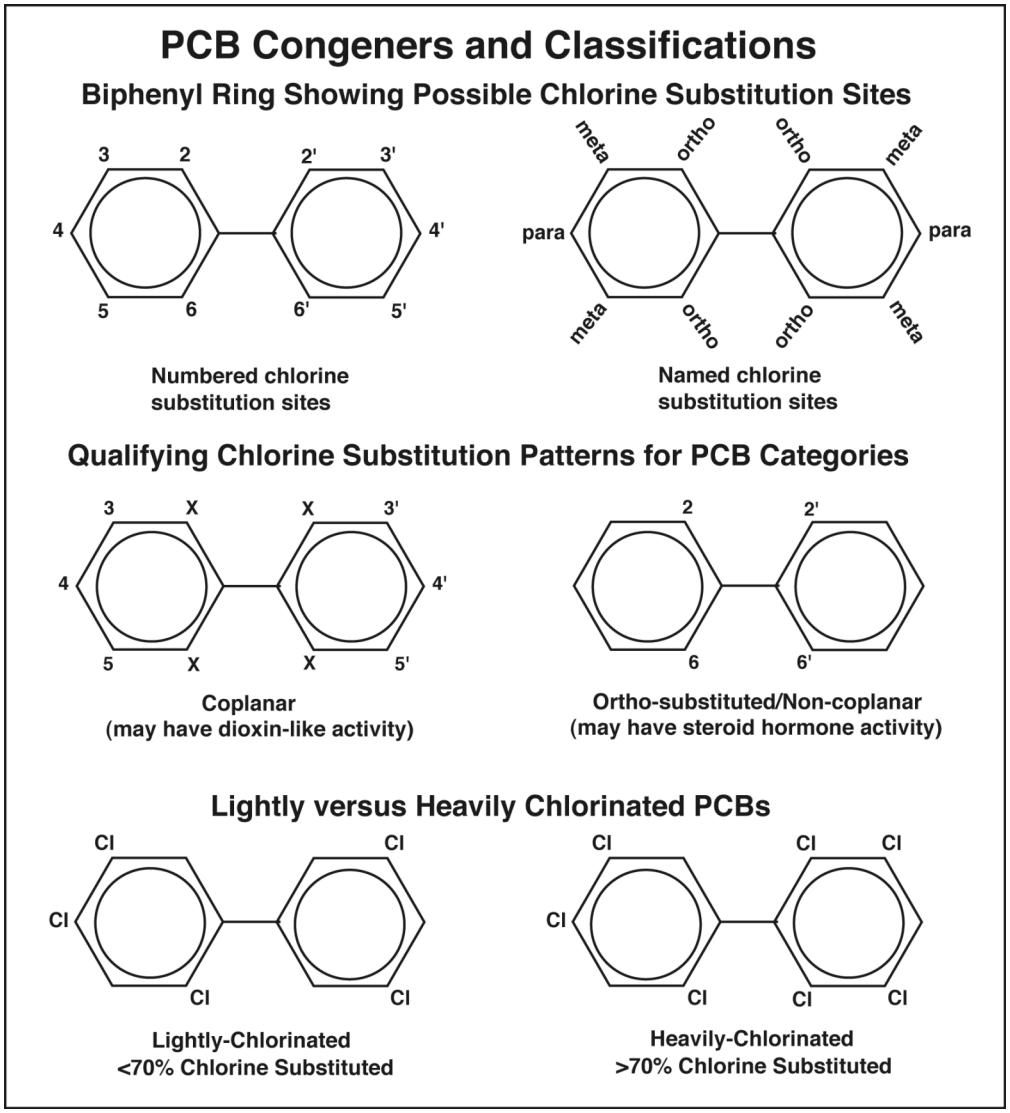


Figure 1: PCBs contain chlorine substitutions at any of 10 available carbons, numbered according to convention. These sites are similarly named according to their relative position to the carbon containing the biphenyl bond: ortho, meta, or para. Coplanar PCBs have a flat 3-D conformation, which requires ≤1 ortho substitution. Noncoplanar PCBs have >1 ortho substitution, resulting in a bend in the biphenyl structure. Some coplanar PCBs can bind to the aryl hydrocarbon receptor (AhR) inducing dioxin-like effects, while non-coplanar PCBs have steroid hormone and neurotoxic effects. Lightly and heavily chlorinated PCBs have different half-lives: heavily chlorinated PCBs endure longer in the environment, and bioaccumulate at a greater rate.

PCBs: ENVIRONMENTAL TOXICANTS

In addition to the extraordinary chemical stability of PCBs, few natural means of degradation exist (Ohtsubo, et al., 2004). Factories and chemical plants may legally dispose of PCBs via flash-incineration at temperatures of at least 1,200 degrees within a 2 second temperature ramp; however, failure to implement these conditions properly can result in the formation of sister compounds with potentially greater toxicity than the PCBs themselves (Weber and Sakurai, 2001). Ignorance or noncompliance of PCB disposal methods has resulted in the improper dumping of PCB waste. In addition, even legally compliant disposed waste receptacles can leak PCBs from landfills into the soil and water. These conditions, compounded over the ~90 years of PCB production, have amassed a wellspring of persistent environmental contamination that extends to animals and humans across the globe.

Primary environmental exposure to PCBs occurs through consumption of contaminated food sources, inhalation, and skin contact. Once inside the organism, PCBs may also be passed from mother to offspring in uterine or oviductal fluids, or in lactate, resulting in transgenerational exposures. Furthermore, lipophilic PCBs are sequestered in fats and other tissues because many organisms lack an adequate catabolic defense against this man-made chemical. Bioaccumulation of PCBs thus occurs through repeated exposure, tissue sequestration, and transgenerational transfer. Although commercial PCB production was halted in 1977, bioaccumulation, continued dumping, and landfill leakage creates a pervasive toxic environment in both urban and rural landscapes across the world. Biotransfer via migratory organisms and oceanic currents has resulted in the paradoxical finding that pristine regions far from primary contamination can contain dangerously high levels of PCBs.

CONGENERS

PCBs form a diverse molecular family because they were manufactured in the form of commercially available isomeric mixtures, each distinguished by degree of chlorination and three-dimensional structure. Now recognized as endocrine disrupting chemicals (EDCs) that can interfere with hormone signaling pathways, PCBs also negatively affect a breadth of other biological systems, including liver metabolism, gastrointestinal function, and adrenal gland function. Early developmental exposure can alter brain patterning, accelerate mammary gland formation, and impair gonadal physiology. In addition to the varied endpoints of exposure, individual PCB congeners affect each endpoint with a unique dose-response curve. Thus, despite the fact that PCBs served a unified purpose in industry, the constituent congeners have very diverse modes of action within a living organism. Biological activity depends on three physical properties of the individual congener: (1) Degree of chlorination, (2) Substitution pattern of chlorine atoms on the biphenyl shell, and (3) 3-dimensional structure of the molecule (Figure 1).

Highly-chlorinated PCBs that contain at least 7 chlorine substitutions have a longer half-life both in the environment, and within living organisms (Platonow and Meads, 1975). They tend to bioaccumulate in the food chain, and certain congeners appear with high frequency in measures of lactate and serum PCB concentrations. Human exposure typically occurs through consumption of intoxicated fishes or meats, or by working directly with PCBs in chemical or manufacturing plants. On the other hand, lower chlorinated PCB homologues are readily metabolized *in vivo*, and thus do not bioconcentrate. These PCBs easily volatize and may be inhaled in aerosol form (Hermanson and Hhites, 1989), or ingested after being

phytoconcentrated in agricultural plant stock (Thomas, et al., 1998a). Aroclor 1221, the low chlorinated commercial PCB mixture employed in this dissertation research, is best known as an estrogenic and carcinogenic mixture (see section: *Aroclor 1221*, under "Exposure Risks"). Low chlorinated PCBs, called "episodic" due to their shorter half-life (Hansen, 2001), are underrepresented in toxicological research, presumably because they are not commonly detected in PCB detection studies of human tissues or in the environment. However, congener-specific measurement methodologies for episodic PCBs have greatly improved in accuracy within the last ten years (Burse, et al., 1996). In addition, recent evidence suggests that women may accumulate the lower chlorinated PCBs to a greater degree than men, either due to dietary habits or to poorer metabolic capacity (Schaeffer, et al., 2006).

Endpoints for Biotoxicological PCB Potencies

The body of research presented in this dissertation has as its goal to investigate the global effect of prenatal PCB exposure on adult female reproduction, focusing particularly on development across two generations, sexual behaviors in the first generation, and gene expression in the first generation. PCBs exert their deleterious effects via a multitude of biological processes, rendering interpretation of these *in vivo* results difficult. Furthemore, natural exposures to EDCs, including PCBs, likely involve complex mixtures, the exact composition of which dependends on the route and site of contamination. For PCBs in particular, multiple congeners originating from different commercial mixtures play a role in exposure. Exposure profiles consisting of individual PCBs along with other environmental toxicants may exhibit additive (Jensen, et al., 2000), synergistic or counteractive (Vettori, et al., 2006) biological effects at certain

concentrations. At the heart of understanding complex mechanisms involving non-linear additive effects of multi-toxicant interactions is a necessarily sophisticated comprehension of the effects of an ecologically relevant exposure profile, such as a single industrial mixture of PCBs. This is the goal of the current series of experiments. In addition, because mechanisms underlying the toxicity of PCBs may act during development to cause permanent alteration of physiological patterning, a thorough summary of PCB exposure endpoints related to this experiment is provided below.

ENDOCRINE DISRUPTION

PCBs may act through many conduits to disrupt animal physiology and behavior. Perhaps the most well documented pathway is through endocrine disruption. Endocrine disrupting chemicals (EDCs), such as PCBs, are natural or man-made chemicals that, when introduced to a natural biological system, disrupt endocrine signaling at the synthesis, transport, signal transduction, or catabolism stages of hormone activity. Lower chlorinated PCBs such as Aroclor 1221, the PCB mixture used in this dissertation, are commonly found to be estrogenic, and higher chlorinated dioxin-like PCBs tend to be anti-estrogenic, however these distinctions are not absolute.

The most highly studied avenue for endocrine disruption by PCBs is steroid hormone interference (Arcaro, et al., 1999, Carlson and Williams, 2001, Moore, et al., 1997). The estrogenic effects of PCBs have been thoroughly investigated. In many cases, the estrogenicity of the PCB mixture is derived from the activity of metabolic breakdown products characterized by hydroxylated carbon moieties (Connor, et al., 1997). Indeed, strong inhibition of estrogen sulfotransferase, an important enzyme for estradiol degradation, was exhibited following hydroxylated PCB

metabolite administration to a human in vitro binding assay (Kester, et al., 2000). Estrogenic and anti-estrogenic PCB congeners and/or their metabolites can bind directly to the estrogen receptor (Connor, et al., 1997, Machala, et al., 2004, Pillon, et al., 2005, Yoon, et al., 2001)(Petit, et al., 1997)(Layton, et al., 2002) and to steroid binding globulin (Kato, et al., 1982), or were identified using an in vitro estrogen-dependent cell proliferation and protein expression assay (Decastro, et al., 2006, Soto, et al., 1995). A 1996 study examining the estrogenic effects of a tetrachlorobiphenyl congener demonstrated PCBs' ability to directly bind the estrogen receptor, induce estrogen receptor binding to gene promoters, induce gene expression, and regulate cell growth in two human breast cancer cell lines (Nesaretnam, et al., 1996). A mixture of a tri- and a tetrachloro-PCB was found to bind to the mouse uterine estrogen receptor in an in vitro binding assay, and to induce expression of estrogen response element (ERE) -dependent genes in a human HepG2 cell line (Ramamoorthy, et al., 1997). *In vivo* studies of juvenile exposure of rats to individual congeners or the commercial PCB mixture A1242 resulted in increased uterine weight and enhanced pituitary response to GnRH, similar to the effects of estradiol administration (Jansen, et al., 1993). PCBs may also act in an estrogenic manner by increasing release of estradiol by female gonads, such as was demonstrated in a porcine follicle culture (Ptak, et al., 2005).

PCBs can alter gonadal steroidogenesis (Grabic, et al., 2006, Gregoraszczuk, et al., 2005, Mlynarczuk and Kotwica, 2005), adrenal steroidogenesis (Li, et al., 2004), gonadotropin levels (Desaulniers, et al., 1999, Lorenzen, et al., 1999) and gonadal hormones. PCBs are also able to bind progesterone receptor (Connor, et al., 1997) and alter progesterone production (Wojtowicz, et al., 2005). In addition to effects on gonadal steroids, PCBs disturb adrenal hormone production and signaling (Goldman

and Yawetz, 1991, Johansson, et al., 1998a, Li and Wang, 2005, Wassermann, et al., 1973). These studies demonstrate that PCBs have the capability to interfere with numerous steroid hormone systems.

Several non-steroid hormones are also affected by PCBs, most notably thyroid hormones, although like steroids, thyroid hormones are also members of the nuclear receptor transcription factor family. Moreover, PCBs are structurally similar to the biphenyl structure of thyroid hormones. PCBs interfere with all levels of thyroid signaling, including transthyretin binding (Purkey, et al., 2004), metabolism (Bastomsky, et al., 1976), and thyroid physiology (Kilic, et al., 2005, Langer, et al., 2003), as well as circulating thyroid hormone levels (Gauger, et al., 2004). Because thyroid hormone signaling is important for normal brain development, PCB depletion of circulating thyroid hormone may cause permanent brain abnormalities (Kimura-Kuroda, et al., 2005, Porterfield and Hendry, 1998, Zoeller, et al., 2000).

In addition to thyroid hormone, serum prolactin levels were depressed following PCB treatment of mice (De Krey, et al., 1994), but were stimulated (Khan, et al., 2002) in rats. Conversely, the PCB mixture A1254 had an inhibitory effect on the prolactin family of genes in rat uterus (Lee, et al., 2003). Coburn et al (2005) found a link between PCB exposure and altered vasopressin release (Coburn, et al., 2005). Similarly, uterine oxytocin production was impaired by PCB exposure in a non-estrogen dependent mechanism (Mlynarczuk and Kotwica, 2005). Thus PCBs have the potential to alter multiple hormone systems.

The secondary effects of endocrine disruption by PCBs include marked effects on female fertility and fecundity. The female reproductive organs are a common target of endocrine disruption. Acute exposure of the uterus results in hypertrophy (Fielden, et al., 1997, Gellert, 1978, Hany, et

al., 1999, Jansen, et al., 1993) whereas developmental and long-term exposures induce uterine hypotrophy (Nixon, et al., 2003, Sager and Girard, 1994). PCBs can alter uterine responsiveness to serum hormones (Sager and Girard, 1994, Tsai, et al., 1997, Wrobel and Kotwica, 2005) and increase the frequency of uterine contractions during pregnancy (Bae, et al., 2001, Loch-Caruso, 2002), which may be related to decreased gestation length in PCB-exposed females. Maternal and paternal exposure to PCBs have been linked with altered sex ratios in humans (Del Rio Gomez, et al., 2002, Hertz-Picciotto, et al., 2003, Mackenzie, et al., 2005, Tiido, et al., 2005, Weisskopf, et al., 2003), whereas wildlife exposure is positively correlated with altered sex ratios in mussels (Hellou, et al., 2003) and turtles (Crews, et al., 1995). PCB exposure in the adult or neonate can alter estrous cycle length in rats (Brezner, et al., 1984, Meerts, et al., 2004b, Sager and Girard, 1994) and alter uterine responsiveness to circulating steroid hormones (Patnode and Curtis, 1994, Sager and Girard, 1994, Tsai, et al., 1997). Therefore, through their interference in proper hormonal communication, PCBs can negatively affect female fertility and fecundity.

NEUROLOGICAL ENDPOINTS AND NEUROTRANSMITTER TARGETS

Though possible explanatory mechanisms are both diverse and poorly understood, PCB disruption of brain functioning is widely supported by the literature (Tilson, et al., 1998). Ortho-substituted PCB congeners (those with a chlorine substitution on carbons 2, 2', 6, or 6'; see Figure 1) appear to have greatest potency in the brain (Shain, et al., 1991). A1221 is approximately 59% mono-ortho substituted, the most potent isomer for brain-related PCB defects. PCB-derived neurotoxicity is hypothesized to be due to any of the following: altered protein kinase C activity (Shafer, et al., 1996), membrane disruption (Tan, et al., 2004a), free radical generation (Mariussen, et al.,

2002), calcium dysregulation (Kodavanti, et al., 1998), or mitochondrial malfunction (Maier, et al., 1994). The cerebellum is particularly sensitive to PCB disruption, which may be related to cerebellar dependence on circulating thyroid hormone during development. Cerebellar granule and Purkinje cell populations are highly susceptible to PCB disruption (Gafni, et al., 2004, Roegge, et al., 2006, Tan, et al., 2004b). Developmentally administered PCBs cause a decrease in cell density of myelinating glial cells in the central nervous system (Sharlin, et al., 2006). They can change cell membrane fluidity and alter the functioning of transmembrane proteins (Tan, et al., 2004a).

Aside from neurotoxic effects, PCBs can alter brain function by changing gene and protein expression, and modulating enzyme activity. PCBs induce early immediate gene expression, c-Jun, in PC12 cells, (Shimokawa, et al., 2006). Expression of synaptic proteins associated with neurodegenerative conditions were affected by PCB exposure (Malkiewicz, et al., 2006). Several neurotransmitter systems have proven susceptible to PCB disruption, notably the biogenic amines dopamine, norepinephrine, and serotonin, and their metabolites. PCB exposure causes intracellular dopamine depletion in a non-AhR dependent manner (Seegal, et al., 1990, Seegal, et al., 1991), but it can also lead to increased dopamine and DOPAC tissue content (Roth-Harer, et al., 2001). Disrupted nitric oxide synthase expression has been postulated as one source of dopaminergic deficiency (Kang, et al., 2002). Decreased serotonin (Chu, et al., 1996, Khan and Thomas, 2000, Morse, et al., 1996b, Seegal, et al., 1986) or norepinephrine (Seegal, et al., 1985) levels are also observed effects of PCB exposure.

PCB-induced disruption of neurotransmitter production and release has been well-characterized for several systems, including acetylcholine, norepinephrine, dopamine, and serotonin. For the cholinergic system,

administration of Aroclor 1254 to adult rats for a 30 day period resulted in decreased activity of the catabolic enzyme acetylcholinesterase (Muthuvel, et al., 2006) due to oxidative damage that was remedied by vitamin C treatment (Venkataraman, et al., 2006). Similarly, activity of choline acetyltransferase, an acetylcholine synthetic enzyme, was depressed in 15 day old rats neonatally exposed to Aroclor 1254 (Juarez De Ku, et al., 1994), and in 30 day old rats perinatally exposed to Aroclor 1254 (Provost, et al., 1999b). The observed interference of PCBs with the acetylcholine system has been proposed to be secondary to thyroid hormone insufficiency (Juarez De Ku, et al., 1994).

Norepinephrine has not been the focus of research investigating PCB effects in the central nervous system. However one study found depressed norepinephrine levels in the hippocampus and frontal cortex of adult male rats exposed to a single large dose of Aroclor 1254 (Seegal, et al., 1985).

Both coplanar and ortho-substituted PCBs can alter the dopamine neurotransmitter system, as reported both *in vitro* and *in vivo*. This appears to be an important target for PCB effects in the brain. An *in vitro* striatal synaptosome preparation exposed to Aroclor 1254 or to individual PCB congeners showed increased media dopamine concentration and DOPAC levels and decreased synaptosomal dopamine (Bemis and Seegal, 2004). Exposure of the same preparation to individual ortho-substituted congeners significantly inhibited activity of the synthetic enzyme tyrosine dehydroxylase, with the strongest effect observed for 2,2' dichlorobiphenyl (Choksi, et al., 1997). In a synaptic vesicle preparation, ortho-chlorinated PCBs inhibit uptake of dopamine into synaptic vesicles (Mariussen, et al., 2001). Data from *in vitro* synaptosomal preparations suggests that PCBs that inhibit dopamine transport into vesicles may not alter transport into the synaptosome, potentially facilitating the accumulation of toxic levels of

dopamine in the cell cytoplasm *in vivo* (Mariussen and Fonnum, 2001). Data from immortalized dopaminergic cell lines suggests that susceptibility to neurotoxicity may also be related to reduced nitric oxide synthase activity (Kang, et al., 2002), or to the induced persistent activity of an enzyme associated with increased production of reactive oxidative species (Lee, et al., 2006).

In vivo studies examining exposures to PCBs demonstrate robust effects on dopamine concentration and metabolism, although they must be considered separately in the context of either developmental or adult exposures. Exposure of adult non-human primates to Aroclor 1016 or 1260 for >20 weeks resulted in decreased dopamine concentrations in the caudate nucleus, substantia nigra, and hypothalamus (Seegal, 1994). Adult rats exposed to 30 days oral administration of Aroclor 1254 exhibited significant decreases in dopamine and its metabolites in the caudate nucleus (Seegal, 1994). A 13-week treatment of weanling rats with the individual congener PCB 28 resulted in depressed dopamine levels in the substantia nigra, with stronger effects observed in females (Caudle, et al., 2006). A study administering PCB 153 to adult rats for 90 days found decreased dopamine content of the frontal cortex, with stronger effects in females (Chu, et al., 1996), and a partner study using PCB 118 likewise found a decrease in dopamine levels of in the substantia nigra of females (Chu, et al., 1995). Amongst a cohort of human factory workers occupationally exposed to PCBs, the incidence of Parkinson's disease, a condition associated with dopamine deficiency, was more prevalent in women (Steenland, et al., 2006). Parkinson's disease is typically more prevalent in men than in women. It is unclear why women were more strongly affected by Parkinson's following PCB exposure, although it may be possible that expression of toxicant metabolism proteins may be altered by exposure to an estrogenic

chemical, such as PCBs (Hernandez, et al., 2006), If this were the case, women exposed to PCBs might exhibit impaired metabolism or increased susceptibility to the neurotoxic effects of PCBs. Another possible explanation is that the estrogenic/antiestrogenic effects of PCBs may subvert the neuroprotective effects of endogenous estradiol in the Parkinson's disease state (Dhandapani and Brann, 2002, Sawada and Shimohama, 2003, Tamas, et al., 2006).

Developmental exposures to PCBs can induce long-term changes in the dopamine pathway that persist through adulthood. Prenatal exposure of rats to Aroclor 1254 resulted in increased dopamine metabolite levels and a higher metabolite : dopamine ratio in male, but not female, adults (Meerts, et al., 2004c). Perinatal exposure of rats to Aroclor 1016 resulted in increased dopamine concentrations in the substantia nigra, caudate nucleus, and nucleus accumbens, with greater effects in females than males (Seegal, 1994). Perinatal exposure of rats to individual coplanar tetrachloro- or hexachlorobiphenyl congeners elevated prefrontal cortex levels of dopamine in the adult (Roth-Harer, et al., 2001). The effects of PCBs on the dopamine system are highly reliant on the age of exposure and the PCB congeners/mixtures encountered (Seegal, et al., 1997).

Finally, PCBs exert actions on the central serotonergic systems. Both developmental and adult exposure to PCBs can alter activity of serotonergic metabolic enzymes and regional brain concentrations of serotonin. Khan and Thomas observed decreased dopamine and serotonin concentrations (Khan and Thomas, 1997) and decreased activity of tryptophan hydroxylase, the synthetic enzyme for serotonin production (Khan and Thomas, 2001), in the hypothalamus of the adult Atlantic croaker following a 30-day exposure to the PCB mixture Aroclor 1254. A similar decrease in tryptophan hydroxylase activity was observed in the brainstem and frontal cortex of the rat following a

single oral dose of Aroclor 1254 (Khan and Thomas, 2001). An increase in serotonin metabolism was observed in telencephalic structures of the rat following either developmental exposure to the coplanar congener PCB 77 (Lilienthal, et al., 1997), or the technical mixture A1254 (Morse, et al., 1996b), and a single oral adult exposure to Aroclors 1254 and 1260 resulted in similar effects (Seegal, et al., 1986).

Thus, PCBs can induce neurotoxic cell death, alter neuronal signaling, and bring about a misdistribution of biogenic signaling molecules in the brain. In the context of the current dissertation work, all of the neurotransmitter systems above play roles in regulating the neural circuits that control reproductive physiology and behavior. Thus, actions of PCBs on central neurotransmitters have direct implications for their effects on the hypothalamic regulation of reproduction.

BEHAVIORAL ENDPOINTS

Behavior is a fundamental endpoint in the examination of low-dose EDC exposures. Because behavior derives from the coordinated contributions of many different neural systems, it is highly sensitive to chemical perturbation. As a counterpart to molecular, cellular, and histopathological research, behavior can reveal new and ecologically relevant information regarding the social and species impact of toxic exposures. The potentially damaging repercussions of PCB exposure on the endocrine system are likely to extend into behavioral abnormalities. Sexual behaviors, in particular, are likely to be influenced by PCB exposure because they are mediated primarily by neuroendocrine brain regions such as the hypothalamus and preoptic area that strongly rely on neurotransmitter and hormone systems known to be targets of PCB disruption.

Because of the many insults exerted by PCBs on endocrine signaling, it is not surprising that adult sexual behaviors are affected. Female rats exposed to PCBs exhibited depressed sexual receptivity (Brezner, et al., 1984, Wang, et al., 2002) and improper timing of mating events (Chung, et al., 2001). Other steroid-mediated behavior such as maternal care are also altered following PCB exposure (Cummings, et al., 2005, Fisher, et al., 2005). Motor activities are a commonly reported symptom of PCB toxicity. PCBs are associated with hyperactivity (Holene, et al., 1998, Lilienthal, et al., 1990), skewed sex-linked sweet preference (Kaya, et al., 2002), and neurobehavioral pathologies (Chou, et al., 1979, Eriksson, et al., 1991). In addition, PCBs can alter sex-typical play behavior in children (Yoon, et al., 2001).

IMMUNO- AND BLOOD DEFICIENCY ENDPOINTS

PCB exposures in humans and animals have been linked with decreased immune potential. Aroclor 1242 and/or 1254, PCB mixtures known to pollute the Great Lakes and Hudson River, can alter ratios of T-cell subsets in mouse (Arena, et al., 2003) and rhesus monkey (Arnold, et al., 1993). Similar immunopathological results have been observed in rat (Sargent, et al., 1991, Smialowicz, et al., 1989). Immune suppression may be coincident with, but does not result from PCB-related increase of serum corticosterone (De Krey, et al., 1993), as circulating corticosterone dampens the immune response potential. Surveys of wild herring gull and harbor seal populations confirm the ecological impact of PCB exposure on immune disfunction (Grasman, et al., 2000, Levin, et al., 2005). Suppression of cytotoxic T lymphocyte production as a measure of the immunotoxic effects of PCBs has been linked with activation of the aryl hydrocarbon receptor (Kerkvliet, et al., 1990, Silkworth, et al., 1984), an orphan nuclear receptor

involved in toxin metabolism. Aryl hydrocarbon receptor (AhR)-independent mechanisms, such as estrogenic effects, may also contribute (Lyche, et al., 2004, Lyche, et al., 2006).

In addition to immune defects, PCB-exposed animals have altered hemotology and serum biochemistry. A1254-exposed rhesus monkeys had decreased erythrocytes and reticulocytes, altered platelet volume, serum cholesterol and total bilirubin (Arnold, et al., 1993), and also displayed hypocellular bone marrow and pathological erythroid precursor cell morphology (Tryphonas, et al., 1986) and impaired erythropoiesis (Tryphonas, et al., 1984). Similar effects, including anemia characterized by low hemoglobin, hematocrit, and red blood cell number were observed in adult PCB-exposed rats (Chu, et al., 1994, Chu, et al., 1998), and low-dose exposures may be effective (Ross, et al., 1996). An in vitro human platelet assay found direct effects of PCBs on platelet activation (Raulf and Konig, 1991). Paradoxical results of increased hemoglobin and red blood cell counts were observed in rainbow trout treated with a PCB technical mixture (Rehulka and Minarik, 2004). Cardiac deformities were observed in passerine birds following developmental exposure to PCBs (Dewitt, et al., 2006). High blood pressure in PCB-exposed humans has been positively correlated with increased serum cholesterol and triglycerides (Stehr-Green, et al., 1986). *In situ* rat and human whole blood assays detected PCB entry into serum lipoprotein reservoirs and erythrocytes, and competitive binding of PCBs to steroid binding globulin and serum albumin (Vomachka, et al., 1983). Additionally, PCBs are associated with vascular endothelial cell dysfunction (Toborek, et al., 1995), perhaps due to PCB-induced oxidative stress (Slim, et al., 1999). Overall, PCBs have great potential to alter an organism's metabolic homeostasis by decreasing the oxygen transport capabilities of the cardiovascular system.

CYTOTOXIC ENDPOINTS

The lipophilic nature of PCBs enables them to pass through plasma membranes, including the cell membrane, endoplasmic reticulum, mitochondrial membrane, and nuclear envelope. PCBs are thus associated with changes in cellular and mitochondrial membrane fluidity (Tan, et al., 2003, Tan, et al., 2004a). Disrupted neuronal membranes could interfere with maintaining ion gradients necessary for neurotransmission (Byrne and Sepkovic, 1987). When this occurs in mitochondria, the phenolic moiety of PCBs can both uncouple and inhibit oxidative phosphorylation involved in energy metabolism (Nishihara and Utsumi, 1987). Related to these cellular processes are PCB disruption of calcium homeostasis, which can lead to the induction of apoptosis; calcium is believed to be released via PCB interaction with intracellular ryanodine receptors (Howard, et al., 2003, Schantz, et al., 1997).

HEPATOTOXICITY

Acute effects of PCBs on liver microsomal activity are well described in toxicology literature. Altered vitamin availability is thought to be induced by dioxin-like PCBs via an AhR-mediated pathway: liver parenchymal cell enzymes are inducted following PCB binding to AhR, thereby exerting direct effects on vitamin metabolism and turnover. Even low-dose exposures to PCBs can deplete vitamin stores greatly needed for the growth and development of the exposed organism (Chu, et al., 1994, Chu, et al., 1998, Kodavanti, et al., 1998).

Vitamin D3 (cholecalciferol) is a steroid-like molecule converted to its active form by the Cytochrome P450 class of enzymes, known to be affected by PCB exposure (Twaroski, et al., 2001). Cholecalciferol's active metabolite

is important for calcium homeostasis, especially in osteoblasts and renal tubule cells, but also in diverse other tissues including the brain. During development, it regulates cell differentiation and proliferation, and can exert transcriptional effects. Lilienthal and colleagues (2000) found that a commercial mixture of PCBs representing congeners found in human breast milk can reduce serum levels of cholecalciferol metabolites (Lilienthal, et al., 2000).

Vitamin A (retinol/retinoic acid) is a powerful morphogen active during development in the differentiation of the nervous system. Decreased hepatic content of retinol is a common marker for PCB exposure, with probable repercussions on retinoic acid signaling (Nilsson, et al., 2000). Liver and serum concentrations of vitamin A (retinol) and retinol binding protein (Innami, et al., 1976) are compromised following exposure. In an example of adult PCB exposure, the accidental contamination of rhesus monkey holding pens with PCBs caused retinol deficiency-related lesions of the glandular stomach and colon (Mcconnell, et al., 1979). Retinol deficiency during development could have repercussions on brain development.

Finally, improper vitamin C and vitamin E levels result from PCB exposures. Vitamin C (L-ascorbic acid) is a powerful antioxidant, and vitamin E (alpha tocopherol) can boost immune function. Increased liver biosynthesis of vitamin C was observed following PCB treatment (Fujiwara and Kuriyama, 1977), and concomitant increased urinary secretion of vitamin C occurs (Horio and Yoshida, 1982). The observed PCB-derived decreases in vitamin E liver content may be related to increased lipid peroxidation resulting from toxicant induction of degradative enzymes (Saito, 1990).

GENE EXPRESSION

Altered gene expression has been observed for several genes following PCB exposure. One mechanism for abnormal gene transcription could be from PCB activation or inhibition of second messenger signaling kinases (Li, et al., 2006, Yang and Kodavanti, 2001), which are involved in transcriptional signal transduction. Some PCBs can alter the post-transcriptional stability of mRNA transcripts (Lin, et al., 2006a), thereby creating a false impression of increased transcription. In addition, PCBs are associated with the increased transcription factor binding of AP-1 to DNA (Twaroski, et al., 2001), and PCB binding to estrogen receptor, thyroid hormone receptor, and AhR induces a transcriptional response (Buterin, et al., 2006, Klinge, et al., 1999, Roelens, et al., 2005, Routledge, et al., 2000, Yoon, et al., 2001). Perinatal exposure to PCBs represses wnt7a transcription factor expression in the developing female uterus (Shimokawa, et al., 2006).

Cancer research suggests the existence of novel genotoxic PCB mechanisms that could play a role in the apoptotic cell death of vulnerable cell populations during development, or could introduce errors into germline stem cells. Some PCBs may derive their carcinogenic qualities from DNA fractionation and the formation of PCB-DNA adducts (Howard, et al., 2003, Mclean, et al., 1996). PCBs are also directly associated with the increased incidence of intrachromosomal recombination (Schiestl, et al., 1997).

HUMAN PATHOLOGICAL RISKS

PCB exposure has been linked to the incidence of several human health risks, including Parkinson's disease, precocious puberty, breast cancer, and poor sperm quality. These correlative studies typically compare plasma concentrations of PCBs with pathological health outcomes. However, the causality of early life exposures to PCBs, and functional outcomes in

adulthood, is extremely difficult to demonstrate. Moreover, some chemical exposures in development may no longer be detectable in blood and/or urine, and results may be difficult to interpret due to non-linearity in the dose-response curve. Finally, many human health studies are confounded by small sample size, and sociological and ethnographic differences in the subject pools, all of which disadvantage researchers from drawing definitive conclusions on the strength of the relationships. Despite these drawbacks, several health risks have been well studied and are generally accepted as relating to PCB exposure.

Because several PCB congeners and mixtures are known to interfere with dopaminergic signaling, researchers investigated the link between PCB body burden and Parkinson's disease, a disorder involving dopamine depletion. A 1998 study found high concentrations of PCBs in dopaminergic brain regions of mortally afflicted Parkinson's patients (Corrigan, et al., 1998). Later research found that PCB concentrations in dopaminergic brain nuclei are specifically associated with Parkinson's and not with other neurodegenerative disorders (Corrigan, et al., 2000), and that women occupationally exposed to high levels of PCBs had an elevated risk of dying from Parkinson's disease (Steenland, et al., 2006), although the small sample size precluded statistical significance.

As an endocrine disrupting chemical, PCBs are known to affect female and male reproductive parameters. Precocious puberty is a recently recognized epidemiological trend for girls to reach puberty at an abnormally young age. PCB exposure has been linked to precocious puberty (Denham, et al., 2005) and *in utero* exposure is associated with abnormal menses (Yang, et al., 2005). In men, measures of human sperm quality have been associated with PCB exposure (Hauser, et al., 2003, Mol, et al., 2002, Spano, et al., 2005, Tiido, et al., 2006). One study including additional

endocrine disrupting chemicals in body burden analysis was able to draw a robust association between PCB exposure and poor sperm quality (Rozati, et al., 2002), and another study investigating the long term health effects of boys exposed to large levels of PCBs *in utero* also found a strong association with poor sperm quality (Guo, et al., 2000).

Several studies have linked PCB body burden with cancer development. The link between PCB exposure and breast cancer is controversial, however recent research uncovered a clear link between genotype and PCB exposure in risk susceptibility for breast cancer (Li, et al., 2005). Additionally, a higher risk of breast cancer was found to be associated with high body burdens of particular PCB congeners (Liljegren, et al., 1998, Lucena, et al., 2001, Moysich, et al., 1998). Prostate cancer is also associated with high tissue levels of particular PCB congeners in vivo (Ritchie, et al., 2003) and in *in vitro* human preparations (Endo, et al., 2003). PCBs have well documented activity on liver toxicity and immune dysfunction in laboratory animals (see sections *Immuno- and Blood Deficiency Endpoints* and *Hepatotoxicity*), and parallel effects have recently been observed in humans (Prince, et al., 2006, Tsai, et al., 2007). Additionally, PCB exposure has been linked with cancer of various organ systems (Pavuk, et al., 2004), perhaps due to its general genotoxicity. A single developmental exposure of male rats to the endocrine disrupting chemical vinclozalin, a fungicidal toxin, can cause a multigenerational increase of susceptibility to a suite of adult-onset diseases (Anway, et al., 2006). More research is required investigating the multigenerational and developmental effects of PCB exposures, both in humans and in animal models.

Large scale PCB exposures have occurred in human populations on two notable occasions: a 1968 incident in Nagasaki, Japan, called “Yusho” in which 1860 people were exposed, and a 1978 incident in Taiwan, called

"Yucheng" in which 2022 people were poisoned. In both cases, PCBs and their related biproducts, polychlorinated dibenzofurans (PCDFs) and polychlorinated quaterphenyls (PCQs), were accidentally introduced into a batch of rice cooking oil. Resulting human exposures were acute and high-dose, and studies focusing on these two unfortunate occasions have provided information on the longitudinal human health effects of both adult and *in utero* or developmental exposures; however, individual PCB or PCDF congeners often cannot be implicated in particular health outcomes.

Both adults and perinatally exposed or postnatally exposed children of the Yucheng incident exhibited skin deformities known as chloracne, abnormal fingernails, skin allergies, goiter, weakened spinal discs, and dental and gum abnormalities (Guo, et al., 1999). Yusho syndrome encompasses chloracne, skin lesions, follicular keratosis, and abnormal pigmentation of the skin, as well as immunocompromised defenses and respiratory infections, fatigue, and headaches (Aoki, 2001). Adults exposed during the Yusho poisoning exhibited chronically elevated levels of serum triglycerides, thyroxin, and IgG in addition to chronic fatigue and numbness (Masuda, 2001). Women exposed in adulthood within the Yucheng cohort exhibited abnormal menstrual bleeding, and high infant mortality of their children (Yu, et al., 2000). Decreased birth weight of children of Yucheng women is hypothesized to be due to altered epidermal growth factor receptor binding, and is correlated specifically with high PCB tissue concentrations (Sunahara, et al., 1987). Adolescent males who consumed contaminated oil in the Yucheng disaster later had a lower probability of producing male children (Del Rio Gomez, et al., 2002). Men exposed in adulthood had a higher rate of mortality from chronic liver disease and cirrhosis and women died more frequently of systemic lupus erythematosus than control populations (Tsai, et al., 2007). Finally, 35 years following the Yusho

poisoning, exposed individuals still exhibit abnormally high levels of oxidative stress believed to be caused by persistent PCB superoxide production (Shimizu, et al., 2007).

Children exposed prenatally to Yucheng PCB-contaminated oils showed retarded behavioral development (Yu, et al., 1994), and achieved lower cognitive measures on standard indices until the conclusion of the study, age 12 (Lai, et al., 1994). In addition, prenatally exposed children were more susceptible to respiratory tract and ear infection, as well as influenza, although no differences in measured immune function were detected (Yu, et al., 1998). Congenitally exposed individuals from the Yucheng poisoning had brittle teeth, often devoid of tooth germ and lower numbers of adult teeth (Wang, et al., 2003) and abnormal skull morphology (Yamashita and Hayashi, 1985). In addition to skin, tooth, cognitive, and behavioral abnormalities, boys exposed prenatally in the Yucheng cohort had shorter penile length, and abnormal sperm morphology (Guo, et al., 2004)(Guo, et al., 2000), Yucheng girls had irregular menstrual cycles and elevated serum levels of estradiol and FSH only in the follicular phase of the estrous cycle (Yang, et al., 2005).

Exposure Risks and Body Burden

Reported levels of contamination for PCBs vary with geographical location and microenvironment, time period, species, diet, subject age, and number of offspring (for females). Estimates of PCB body burden in humans canunderestimate total body burden when a subset of congeners commonly found in human tissues is chosen to represent all PCBs. Additionally, tissue levels of PCBs within organs, skin, and bone are not accounted for. In human studies, plasma levels of PCBs are commonly utilized as a proxy for total PCB body burden.

Stellman et al, 1998, found that in human women 10 ng/ml total PCBs in blood serum corresponds to 1 mg/kg adipose tissue (Stellman, et al., 1998) and this conversion factor will be used as an approximate measure to equilibrate different metrics. Focusing on Long Island women, considered a high risk group for PCB exposure, Stellman *et al* documented adipose levels of PCBs ranging from 0.27 to 1.1 mg/kg. In 1991 the FDA estimated average daily dietary intake of PCBs to be less than 1 ng/kg/day (Gunderson, 1995), although mean serum levels of PCBs in male Great Lakes sports fisherman with a high fish diet was 4.8 ng/ml (~0.5 mg/kg adipose PCBs) (Hanrahan, et al., 1999). Inuit peoples with a diet high in fish and marine mammals have plasma PCB/lipid concentrations up to 1.1 ppm, which is equivalent to 1.1 mg/kg serum PCBs and suggests far higher levels of adipose PCBs (Carpenter, et al., 2005). In the lower risk population of Western Germany, human neonatal babies consumed approximately 1.6 μg/kg/day PCBs from breast milk in 2003 (Furst, 2006).

Animals at greatest risk of toxic effects of PCB exposure typically inhabit marine environments, where PCBs are highly concentrated through bioaccumulation up the food chain, and atmospheric transport results in rain deposition. PCB levels are highest in marine mammals, with recorded levels of 29-79 mg/kg adipose concentrations within Beluga whales of the St. Lawrence River (Muir, et al., 1996). Sea birds are also exposed to high levels of PCBs, and are particularly sensitive to PCB toxicity; 1183 ng/g PCBs in plasma lipids (~1.2 mg/kg serum PCBs) was measured in Arctic glaucous gulls (Verreault, et al., 2007). Farmed salmon from Maine were measured to have 0.15 mg/kg PCBs per lipid weight (Shaw, et al., 2006). Herbivorous land-locked species such as caribou provide a useful reference for monitoring environmental transfer of PCBs transported via volatization and atmospheric circulation rather than bioaccumulation. Adipose PCB

concentrations in Canadian caribou populations ranged between 0.006 and 0.032 mg/kg PCBs in 1995 (Elkin and Bethke, 1995).

DEVELOPMENTAL EXPOSURE

Adult exposures to low doses of EDCs typically result in significant, time-delineated, alteration of hormonal signaling (Ahmad, et al., 2003, Desaulniers, et al., 1999, Khan and Thomas, 2001). Gradual recovery follows removal of the aggravating chemical agent and thus the effects of exposure, while in some cases grave, represent transient modifications on a fully developed system. In contrast, EDC exposures in the embryo or fetus can redirect the intended structure of the nervous system, forming a permanently altered network. This is because steroid hormones play an organizational role in the neuroendocrine brain, and disruption of neuronal patterning manifests itself as a disfunction when these systems are activated in the mature adult. The biphenyl structure of PCBs and other EDCs resembles submolecular regions of hormones that are involved in receptor binding. This structural similarity allows them to mimic or antagonize intrinsic steroid hormones in multiple systems. When hormonal disruption occurs in early development, EDCs have the capacity to alter formative processes, such as neuronal pathway formation, cell migration and survival, and receptor gene and protein expression, each of which may chronically affect brain function and/or brain sexual differentiation.

In utero exposure to PCBs has far greater potential to induce enduring negative effects than adult exposure. Although exposure during adulthood may result in short-term perturbation of neuroendocrine systems (Richardson and Miller, 2004, Seegal, et al., 1985), behavior was not strongly affected (Chung, Nunez, Clemens, 2001). On the other hand, perinatal exposure to PCBs can repattern the susceptible embryonic brain, resulting in the

eventual manifestation of improper sexual behaviors (Chung & Clemens 1999: Sager, 1983) and hyper- or hypoactivity (Jacobson JL et al, 1992: Hany et al 1998). There may be differential vulnerability between the sexes, depending upon age and mode of exposure, and the type of EDC. The female mammalian embryo may be particularly sensitive to the estrogenic effects of PCBs because she is normally shielded from maternal steroidal hormones by alpha-feto binding protein, and produces only trace endogenous hormones in highly localized tissues. By contrast, the fetal male testis is quite active and exposes the developing male organism to much higher levels of steroid hormones. It might be predicted that for some of these endpoints in the male, exogenous hormones may be less deleterious than in the female, in which endogenous hormone levels are naturally far lower. Circulating steroid hormones in development can effect a permanent repatterning of the plastic embryonic brain, and this effect is mimicked by endocrine disrupting chemicals.

AROCLOR 1221

This study examines the behavioral effects of transplacental and lactational exposure to the commercial PCB mixture Aroclor (A)1221. Previous studies have demonstrated the potential for Aroclor A1221, or its constituent congeners, to interfere with puberty and reproductive aging (Gellert RJ 1978), increase uterine weight (Ecobichon and MacKenzie 1974), alter neurotransmitter signaling (Seegal et al, 1990), and interfere with hormone signaling (Gore et al 2002, Kilic et al 2005, Layton et al 2002, Woodhouse et al 2004), all of which are involved in the exhibition of mature sexual behaviors.

A1221 is a low (21%) chlorinated PCB mixture believed to have a short half-life, high metabolic clearance, and estrogenic activity. Its

estrogenic effects are likely primarily mediated by the hydroxylated or catechol metabolites created during enzymatic breakdown (Korach et al, 1987, Kramer et al 1997, Kitamura et al, 2005, Garner et al, 1998, Connor et al 1997, Layton et al, 2002), but non-steroidal interactions may involve the parent molecule (Brouwer et al, 1999). Aroclor 1221 can activate estrogen receptor-dependent activity in an *in vitro* preparation of preneoplastic breast epithelial cells and breast cancer cells (Shekhar, et al., 1997). One major constituent of A1221, 4-chlorobiphenyl, and its metabolites, induce estrogen release from preovulatory ovarian follicles up to 256 fold greater than control levels *in vitro* at a 6 ng/ml concentration (Ptak A et al, 2005). Constituent hydroxylated members of A1221 were shown to bind estrogen receptor alpha in a modified recombinant yeast estrogen assay (Layton, et al., 2002). A1221 inhibits the ability of aromatase enzyme to convert testosterone to estradiol in a commercially available microsomal fraction transfected with human CYP19 and P450 reductase (Woodhouse and Cooke, 2004).

Because of its presumed short-lived tissue contamination before anabolic breakdown, A1221 has not recently been the focus of toxicological research. However, some recent findings demonstrate the potential for A1221 to alter neuroendocrine gene expression (Salama, et al., 2003), and induce chromosomal recombination (Schiestl et al, 1997) that is linked with cancer and could alter germline heredity. In addition, this mixture contains a major di-ortho substituted congener known to cross the blood-brain barrier and interfere with catecholaminergic neurotransmission (Seegal, et al., 1991)(Frame, 1997). Furthermore, prior studies documenting relatively low levels of environmental contamination by A1221 should be reevaluated in light of novel, more refined analyses (Frame, 1997: Becker et al, 2002). In all, the sum of previous research demonstrates the potential for lightly chlorinated PCBs such as A1221 to greatly affect normal physiology and

dramatically alter the neuroendocrine axis of the unborn embryo, acting quickly and catastrophically before being cleared from the body.

Leakage of A1221 from waste dumps is well-documented (Agency for Toxic Substances and Disease Registry, 2000), and the potential for animal and human exposure exists from inhalation of airborne particles, in drinking water, and through fish consumption from intoxicated areas. In addition, previous contamination analysis relied on relatively insensitive measures and/or used a single marker congener to represent levels of the entire PCB commercial mixture. Accurate quantification of specific congeners comprising A1221 can be difficult and expensive, and methods quoted prior to 1997 are believed to have inferior resolution compared to recent, improved assays (Frame, 1997: Becker et al, 2002). Human and wildlife contact with A1221 may be underestimated, evidenced by a paucity of peer-reviewed studies on low-dose exposures.

Table 1 shows the breakdown by weight of A1221 into each of its constituent congeners by electron capture detector (ECD) adapted from G.M. Frame, 1996. The Aroclor 1221 stock solution analyzed by Frame, 1996, is lot # 073-202 from Accustandard, Inc., and the stock used in the current dissertation research is lot # 072-202, from the same company. As the Frame, 1996, study represents the most thorough reporting to date of A1221 constituent congeners, and because Accustandard Aroclors are the industry standard and are accepted as highly reliable from lot to lot (personal communication, Dr. Richard Bopp, Rensselaer Polytechnic Institute), the contituent member congeners of A1221 given in Table 1 (page 31) are believed to represent the treatment administered in this dissertation research.

The biological impact of lightly chlorinated congeners such as those that compose the majority of A1221, by mass, is not well-studied. For

example, while PCB 1, 2-chlorobiphenyl makes up ~31% of A1221 (see Table 1), no data could be found on its toxicological effect. I will begin this discussion on those congeners of A1221 with poorly understood effects, and follow in the next paragraphs with information about the better-investigated congeners, PCBs 3 and 4. PCB 2 and PCB 3, effects of which are not well-documented, are associated with elevated production of reactive oxidative species in rat synaptosomes (Voie and Fonnum, 2000). PCB 2 has been shown to quickly transfer amongst the three densities of human plasma lipoproteins in blood (Maliwal and Guthrie, 1982). PCB 15, also referred to as 4,4'-dichlorobiphenyl, is associated with genetic recombination events (Butterworth, et al., 1995). PCB 18 is not well-studied, however it has recently been documented in higher than expected concentrations in native American women (Schaeffer, et al., 2006).

PCB 3, 4-chlorobiphenyl, is one of the most well-studied constituents of A1221, and comprises approximately 16% of the mixture, by mass (Table 1). It is associated with cellular stress in a bacterial model (Agullo, et al., 2007). Environmentally-relevant exposures to PCB 3 induce apoptotic gene expression and genotoxicity (Ptak, et al., 2006a) and can increase aromatase activity and decrease progesterone secretion (Ptak, et al., 2006b) in cultured porcine ovarian granulosa cells. Genotoxicity by PCB 3 is believed to be induced by the formation of DNA adducts (Mclean, et al., 1996), although PCB 3 has also been shown to decouple and inhibit oxidative phosphorylation during cellular metabolism (Nishihara and Utsumi, 1987).

PCB 4 is an *ortho*-substituted PCB congener also known as 2,2'-dichlorobiphenyl, and it makes up approximately 6% of A1221. *In vitro* uterine explants from 10 day pregnant rats exhibited an inhibition of contraction amplitude and desynchronized contractions following exposure to

PCB 4 (Chung and Loch Caruso, 2005). Because it is ortho-substituted, PCB 4 is attributed with increased permeability into the brain, and neurologically damaging effects, such as nitric oxide synthetase inhibition (Sharma and Kodavanti, 2002), induction of intracellular calcium fluctuation in cerebellar granular cells (Bemis and Seegal, 2000), alteration of PKC second messenger signaling (Yang and Kodavanti, 2001), and gross neurotoxicity (Shain, et al., 1991).

Table 1: % Congener content of Aroclor 1221 by weight, via high resolution gas chromatography (HRGC) and electron capture detection (ECD) *adapted from (Frame, 1996)*

PCB	IUPAC name	Cl- substitutions	PCB 3D shape	% weight
0	Biphenyl	None	coplanar	12.0
1	2-chlorobiphenyl	mono-orthosubstituted	coplanar	31.5
2	3-chlorobiphenyl	non-orthosubstituted	coplanar	3.4
3	4-chlorobiphenyl	non-orthosubstituted	coplanar	18.0
4	2,2'-dichlorobiphenyl	di-orthosubstituted	non-coplanar	5.4
5	2,3-dichlorobiphenyl	mono-orthosubstituted	coplanar	0.65
6	2,3'-dichlorobiphenyl	mono-orthosubstituted	coplanar	3.4
7	2,4-dichlorobiphenyl	mono-orthosubstituted	coplanar	1.5
8	2,4'-dichlorobiphenyl	mono-orthosubstituted	coplanar	10.9
9	2,5-dichlorobiphenyl	mono-orthosubstituted	coplanar	1.5
10	2,6-dichlorobiphenyl	di-orthosubstituted	non-coplanar	0.7
11	3,3'-dichlorobiphenyl	non-orthosubstituted	coplanar	0.1
12	3,4-dichlorobiphenyl	non-orthosubstituted	coplanar	0.5
13	3,4'-dichlorobiphenyl	non-orthosubstituted	coplanar	1.0
15	4,4'-dichlorobiphenyl	non-orthosubstituted	coplanar	3.7
16	2,2',3-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0.3
17	2,2',4-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0.3
18	2,2',5-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0.7
19	2,2',6-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
20	2,3,3'-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.1
22	2,3,4'-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.2
25	2,3',4-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.1
26	2,3',5-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.1
27	2,3',6-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0
28	2,4,4'-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.5
31	2,4',5-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.5
32	2,4',6-trichlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
33	2,3',4'-trichlorobiphenyl	mono-orthosubstituted	coplanar	0.4
37	3,4,4'-trichlorobiphenyl	non-orthosubstituted	coplanar	0.2
42	2,2',3,4'-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
44	2,2',3,5'-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.2
48	2,2',4,5-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
49	2,2',4,5'-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
52	2,2',5,6'-tetrachlorobiphenyl	tri-orthosubstituted	non-coplanar	0.2
56	2,3,3',4'-tetrachlorobiphenyl	mono-orthosubstituted	coplanar	0.1
60	2,3,4,4'-tetrachlorobiphenyl	mono-orthosubstituted	coplanar	0.1
64	2,3,4',6-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
65	2,3,5,6-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
66	2,3,4,4'-tetrachlorobiphenyl	mono-orthosubstituted	coplanar	0.2
70	2,3',4',5-tetrachlorobiphenyl	mono-orthosubstituted	coplanar	0.2
71	2,3',4',6-tetrachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
74	2,4,4',5-tettrachlorobiphenyl	mono-orthosubstituted	coplanar	0.1
101	2,2',4,5,5'-pentachlorobiphenyl	di-orthosubstituted	non-coplanar	0.1
118	2,3',4,4',5-pentachlorobiphenyl	mono-orthosubstituted	coplanar, dioxin	0.1

A large percentage of the constituent congeners of A1221 are ortho-substituted, which endows this commercial mixture with a certain amount of versatility in its effects: (1) mono-ortho congeners are able to maintain a flat coplanar conformation (U.S. Environmental Protection Agency (EPA) Table of PCB Congeners & Other Species; http://www.epa.gov/toxteam/pcbid/table.htm#key), (2) certain hydroxylated metabolites can enact the bent conformation required for agonistic mimicry at the estrogen receptor (Layton, et al., 2002), and (3) di-orthosubstituted congeners are efficacious at crossing the blood-brain barrier and enacting neurotoxicological effects (Angus and Contreras, 1996, Maier, et al., 1994, Seegal, et al., 1991). This suggests that A1221 has the potential to perturb both steroid hormone and neuronal systems.

Aroclor 1221 has the lowest chlorine content of any of the commercial PCB mixtures made available in the United States. Lower degrees of chlorination correlate with decreased longevity compared with the more highly chlorinated congeners. Thus PCBs such as A1221 are quickly broken down into their hydroxylated and sulfylmethylated metabolites. Despite its proven carcinogenicity, endocrine disrupting effects, and thryotoxicity, A1221 is not a common choice for toxicological research. Methodological difficulties arise from the fact that detection of A1221 is difficult and costly compared to measurements of the higher profile, more heavily chlorinated mixtures. Additionally, body burden of A1221 declines in a matter of days following exposure due to compensatory xenobiotic metabolism.

Other PCBs, laden with chlorine substitutions, are heavy and highly lipophilic. They are forced out of industrial waste sites by rainwater and erosion, and accumulate in the microscopic organism reservoir at the sediment layer of waterways, lakes and oceans. Their lipophilicity and long half-life of years to decades facilitates their escalating accumulation at each

ream of the food chain, until they appear in measurable quantities in humans and upper trophic-level predators typically exposed via seafood. A1221, however, tells a different story. It is light and ephemeral, often volatile. It enters a gaseous state in the air above superfund industrial dumps, or in the ducts of old buildings outfitted with grandfathered circulation systems. In this state, it can be globally transported in the upper layers of the atmosphere and deposited in remote locations where it can be broken down by bacteria. Most importantly, however, A1221 can be inhaled or absorbed directly by humans and wildlife, or absorbed by agricultural plant stock and later consumed.

A1221, together with its metabolites, presents a special challenge to epidemiological studies of environmental toxicology. Exposure is quick, followed by the rapid production of arguably more toxic catabolic derivatives, and then within weeks tissue levels decline below measurable quantities. To formulate judgements on the health impacts of PCBs, most medical doctors and researchers have focused on heavily chlorinated PCB congeners that persist in human tissues, and can be extracted from cancerous cysts or placental tissues. While this approach is valuable, it largely ignores the dangerous effects of lower chlorinated PCBs, which at very low levels can create a grave disturbance in the physiology of exposed organisms – particularly during sensitive periods of embryonic development – and then practically disappear.

NON-LINEAR DOSE-RESPONSE CURVES

Understanding the low-dose effects of environmental toxicants is crucial to human health and wildflife survival (Kuriyama & Chahoud, 2005). This is especially true for EDCs. Because of their ability to act as, or interfere with, endogenous hormones, EDCs elicit a hormone-typic dose response curve

shaped like a “U” or “inverted U” (Calabrese and Baldwin, 2001a). In this case, a median dose results in the most dramatic effects on the biological endpoint being measured and doses at the extremes of the dose-response curve have a lesser magnitude effect. Steroid hormones such as estradiol often exhibit complex, non-linear dose response curves shaped by complex interactions with other hormones and mediated by different nodes of the signaling circuit. Examples include the uterotrophic effect of estradiol (Barton and Andersen, 1998), estradiol-dependent down-regulation of uterine estrogen receptor alpha (Medlock, et al., 1991, Medlock, et al., 1994), and the effects of estrogen on GnRH-mediated LH release from pituitary gonadotrophs (Yen and Lein, 1976). Non-linear, or U-shaped dose response curves might also occur on a molecular basis for transcriptionally active nuclear receptors, such as the estrogen receptor, when endogenous ligand is not present in saturating concentrations and binding by the xenobiotic does not produce an ideal receptor conformation for cofactor binding (Kohn and Melnick, 2002). The introduction of exogenous estrogenic substances to a hormone-driven system produces effects that fall within the intrinsic non-linear dose-response relationship (Sheehan, 2000).

Another possible explanation for the non-linear curve is an effect known as hormesis, in which an organism or system overcompensates for the negative impact of a toxic insult; at the low end of the dose-response curve, compensation is not enough to overcome toxic effects, but at median levels of intoxication, overcompensation of the system results in an opposite magnitude of effect. At high doses, the toxicant may again overcome the system, resulting in another reversal in the direction of effect. Whereas a steroid hormone-like effect is mediated by interaction between a hormone mimic and a steroid hormone enzyme or receptor, hormesis is a more generalized response to overt toxicity. The prevalence of non-linear dose-

response curves in toxicological research underlies the need for assaying a range of doses in PCB research.

LOW-DOSE EXPOSURES

Many prior studies examining the effects of PCBs *in vivo* employed high doses not normally encountered by humans or animals, but low dose exposures during embryogenesis have the potential to induct permanent or long-term aberrations in the adult. For example, low doses of A1254 administered prenatally to rats resulted in severely depressed circulating thyroid hormone levels in postnatal and adult offspring (Morse, et al., 1996c), whereas far greater levels are required for similar effects in the adult (Brouwer, 1989). In addition, low versus high exposures may be associated with unique effect profiles at the organismal level. High levels of PCBs may have increased pervasiveness in less accessible tissues, such as remote brain regions, and could recruit cytotoxic mechanisms unique from their low-dose counterpart (Brouwer, et al., 1999). Thus, studies reporting only atypically high EDC exposures may be misleading for predicting response to ecologically relevant exposures. The results of high-dose toxicology research may therefore be largely distinct from low-dose exposures.

Goals of this Research

My dissertation studies were designed to investigate the global effects of PCB exposure on females during the embryonic period of brain sex differentiation. Because of the expected U- or inverted-U shaped dose-response curve, a range of dosage values encompassing typical human PCB tissue burdens was chosen to determine toxic exposure levels. In addition, previous research examining PCB effects on mating behaviors employed ovariectomized, hormone replaced females, a paradigm which conceals the

subtle effects of PCB-related alterations in circulating hormone levels by replacing them with standard hormone doses. Finally, I attempted to elucidate some of the molecular mechanisms and molecules underlying the effects of PCBs on reproductive and behavior by pursuing a whole-genome approach with targeted gene expression follow-up. Thus, the following work describes the effects of prenatal A1221 during a period of brain sexual differentiation on development and reproductive hormone parameters across two generations, adult female reproductive behaviors, and gene expression in a neuroendocrine region of the brain.

General Methods

To avoid redundancy, a general description of methods is provided here with background information, and more precise details are given in each of the three research sections.

Animals and Husbandry

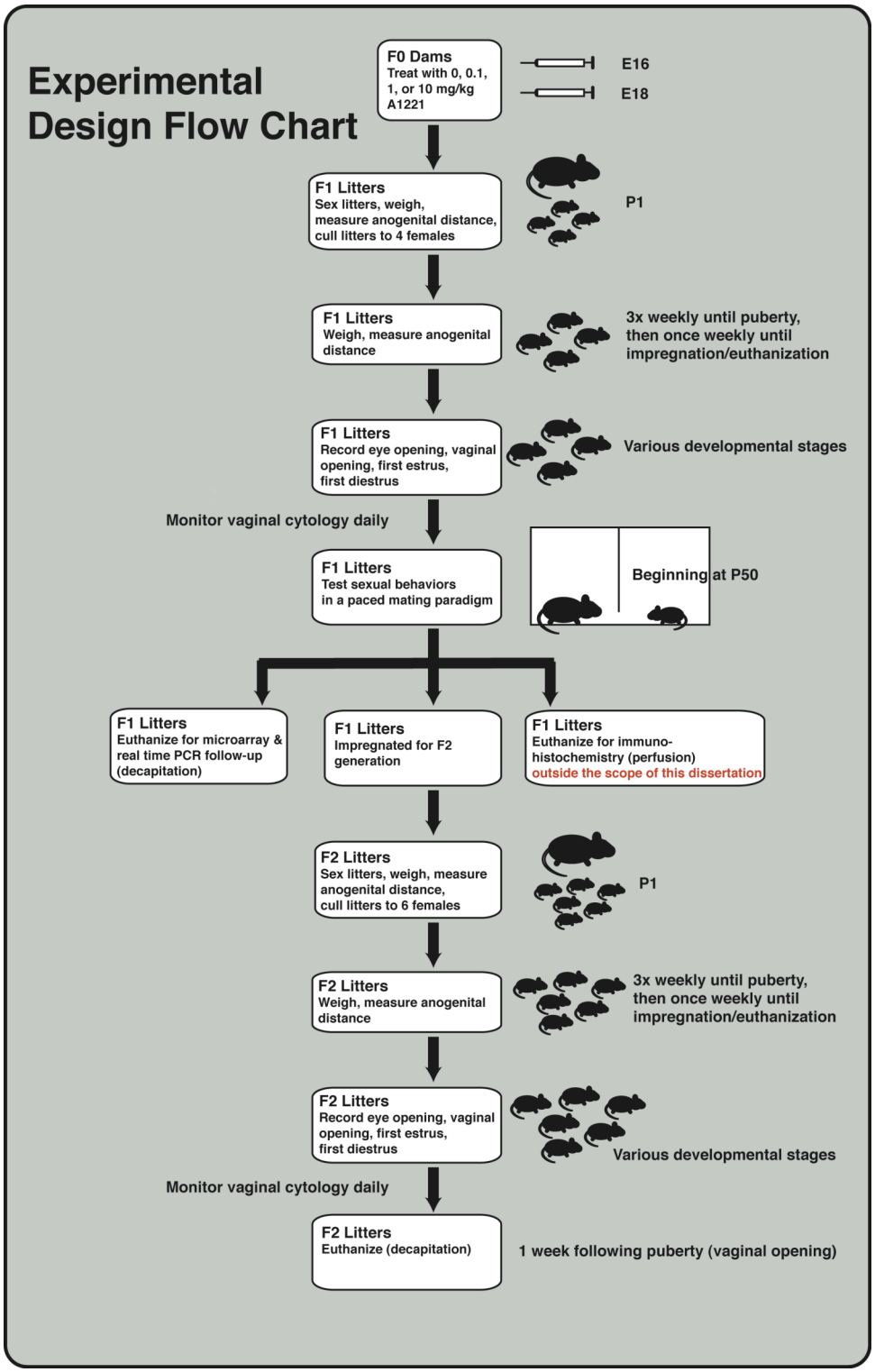
Sprague-Dawley rats were used for all experiments. Rats were kept on a 12:12 hour partially-reversed light cycle (lights on at 2300H) and were given the low-phytoestrogen Harlan-Teklad Global Diet 2019 rat feed, and water, *ad libitum*. One-day timed-pregnant nulliparous females (F0 generation) were purchased from the Animal Resources Center at the University of Texas at Austin. The evening of successful mating is termed embryonic day (E) 0. F0 dams were housed individually, and were weighed on E15 to calculate treatment dosage: either vehicle (DMSO), 0.1, 1 or 10 mg/kg PCBs with a total injection volume of 0.1 ml. This range of PCBs was chosen to span ecologically relevant human and animal exposures (see section Exposure Risks). Aroclor (A) 1221 PCBs were diluted in DMSO, and stored in a single 5 ml Falcon tube (to prevent PCB adsorption to the novel tube every time a new stock solution was made). This stock solution was kept light-protected at room temperature. Injections were delivered intraperitoneally on E16 and E18 one hour prior to lights-out. Because A1221 has a low half life in vivo, and because experimental animals were euthanized long after exposure, we were unable to measure body burden and brain content of A1221, however in future experiments several neonatal pups will be reserved to measure these parameters. On E20, dams were administered nesting materials, and were not disturbed until postnatal day

(P) 1 to prevent stress that may interfere with parturition or maternal behaviors.

Developmental measures of first generation (F1) offspring

A flow chart depicting allocation of animals to each branch of the study is provided in Figure 2. P0 was counted as the day that at least one pup was born before lights out at 1100H. On P1, pups in each litter were counted, dead pups noted, sex ratios (including dead pups) were recorded, and all offspring were weighed and their anogenital distance measured an indicator of exposure to prenatal androgens (Gray, et al., 2001). In addition, nest quality was qualitatively described and the dams were checked to see if they fetched their pups within 5 minutes, although these qualitative observations of maternal behavior were not used in analysis. Litters were culled to 4 females, or fewer when 4 were not available; these experimental females were chosen to have a median anogenital distance normalized to body weight, to eliminate bias caused by fetal proximity to a male, which can influence anogenital distance (Vom Saal, 1989). Body weights and anogenital distance were taken throughout development on the following timepoints: P1,3,5,7,9,13,16,20,23,27. Pups were weaned on P22 to 3-4 littermates/cage. Developmental landmarks of eye opening and vaginal opening were recorded. Following vaginal opening, daily vaginal smears were conducted to determine estrous cyclicity, and body weights were measured once per week. More detailed information may be found in Research Section 1.

Figure 2: Experimental Design Flow Chart



Paced mating reproductive behavior testing of F1 females

Beginning on day P50, females were submitted to a paced mating protocol to test the effects of PCB exposure on female sexual behaviors. Paced mating cages were constructed of a 30"Lx12"Wx17"T Plexiglas aquarium fitted with a clear Plexiglas panel bisecting the cage lengthwise. Two 1.75" diameter holes at the base of this panel allow the female to pass between the two chambers, however, males were too large to fit. Males were untreated with PCBs, and were sexually experienced. Ultrasonic noise sources were located with a bat detector and removed from the vicinity of the experiment. Mating trials were conducted 5 hours after lights out under dim red lighting, on the evening of proestrus (behavioral estrus). Female and male rats were habituated to the mating cage at least three times prior to the experimental trial, plus for at least 10 minutes on the experimental day. Females were given 20 minutes to display receptivity, or else the trial was called "unsuccessful" and the female was returned to her cage. Females were allowed 4 different attempts to mate before being eliminated from the experiment. In all cases females were tested only when they had a vaginal smear indicating proestrous reproductive status on the morning of the mating trial.

Successful mating trials consisted of at least 7 intromissions and at least 1 ejaculation. Ejaculation was confirmed by the presence of sperm in the vaginal smear. Successful mating trials were recorded on video camera, and were later reviewed and transcribed into an events log for analysis, with the investigator blind to treatment. The following behaviors were scored: mounts, intromissions, ejaculations, lordoses, lateral kicks, face-to-face's, cage-crossings, and audible vocalizations. Further methodological details are provided in Research Section 2.

Assignment of F1 females after mating

The day following a successful mating trial, F1 female rats were divided into three groups (Figure 2). Of the four females per litter, one was kept to produce a second generation; two females were decapitated for molecular analysis, and the final female was perfused for future protein expression analysis (beyond the scope of this dissertation). Euthanasia was performed at ~10:00 AM (1 hour prior to lights out, and approximately 20 hours following mating). On the day of euthanasia, vaginal smears and body weights were recorded. After euthanasia, ovaries and uteri were removed, and their wet weights recorded (uterus alone, then both ovaries together). Females euthanized via decapitation also had trunk bloods taken so that the serum could be stored for subsequent serum hormone analyses.

F2 generation: husbandry and procedures

As stated above, one F1 female per litter was allowed to carry the litter to term after the paced mating trial. Thereafter, impregnated F1 females were submitted to identical conditions as the F0 dams, except that they did not receive any further administrations of the PCBs. Following parturition, the F2 generation was treated identically to the neonate F1 generation with the exception that six female pups were kept per litter, when possible, instead of four. F2 experimental females were euthanized one week following vaginal opening at approximately P42 for serum hormone assays and determination of uterine and ovarian weights. F2 rats were virgins at the time of euthanasia. Further description of studies on the F2 generation is provided in Research Section 1.

Serum hormone assays

Blood samples collected at the time of euthanasia were centrifuged at 6000 x g for 5 minutes, and serum of F1 and F2 female rats was removed and stored at -80° C until analysis. Serum hormone levels were assayed by radioimmunoassay (RIA) for luteinizing hormone, estradiol, and progesterone concentrations. Serum LH for each animal was assayed in duplicate samples (50 μl) by Dr. Michael J. Woller (University of Wisconsin-Whitewater), using a radioimmunoassay (RIA) with LH anti-serum (NICHHD R13, pool D; provided by Dr. G. Niswender). Serum estradiol (E2) was measured in duplicate via the Diagnostic Systems Laboratories DSL-43100 estradiol RIA kit, according to the kit protocols across a total of five assays. Serum progesterone (P4) was measured in duplicate via the DSL-3900 progesterone RIA kit, in accordance with kit protocols across a total of 8 assays. Outliers were eliminated from analysis (see statistics section, below).

RNA extraction for molecular biology procedures

RNA was extracted using a cushion/lysis buffer method (Daftary and Gore, 2003, Gore and Roberts, 1994, Gore, et al., 1999): Frozen POA tissues were homogenized via extrusion through a 22 gauge needle, proteinase K was introduced, and then RNA was extracted in phenol chloriform and precipitated in ethanol. Nuclear and cytoplasmic RNA were separated using a two buffer system. Namely, frozen tissues were homogenized in a 22 gauge needle in lysis buffer (containing Na+ Deoxycholate). A centrifugation step allowed cytoplasmic RNA to remain in the supernatant, a high molarity solution of salts and sugars, whereas nuclear RNA pellets at the base of the tube. Cytoplasmic RNA was carefully pipetted away from the pellet, and treated with proteinase K to eliminate cytoplasmic proteins. RNA was further purified through a series of

phenol/chloriform extractions in which hydrophobic constituents were separated from the aqueous supermatant, and finally, RNA was separated from solution with isopropanol. The RNA pellet was then dried and reconstituted with nuclease free water. Extracted RNA was frozen at -80 degrees prior to amplification. The cytoplasmic RNA of 4-5 animals per treatment, each representing a different litter, was chosen for microarray analysis (For control, 0.1, 1, and 10 m/kg groups, n/treatment group were 5,4,5, and 4, respectively).

Affymetrix 230 2.0 rat whole genome array

The Affymetrix rat 230 2.0 whole genome array is a valuable tool for measuring gene expression changes in pharmacogenetic studies. This microarray chip is composed of a grid of oligonucleotide cRNA probes (25 base pairs (bp) each), and each "space" on the grid is occupied by multiple copies of identical probes. Because it is a whole genome GeneChip, all known RNA transcripts from the rat genome are represented. Over 31,000 transcripts, including isomers and splice variants, make up the array, representing 28,000 genes. Introduction of amplified RNA (aRNA) from an experimental sample causes binding at each probe location, resulting in formation of a fluorescent double-stranded reaction product. Eleven to twenty "perfect match" (PM) probes are designed for each transcript, and to control for false positive results, an equal number of paired mismatch (MM) probes are included in parallel, which include a single incorrect bp in the center of the probe (bp 13). Signal intensity from MM probes may then be subtracted from that of PM probes to control for nonselective binding. Each probe is synthesized *in situ* on one space of the etched photolithographic glass of the microarray plate, in an approximately 14 μl diameter well.

RNA amplification & microarray hybridization

Cytoplasmic RNA of samples chosen for microarray was submitted to Ambion, Inc. (Austin, TX) for Affymetrix Rat 230 2.0 whole genome GeneChip hybridization. Ratio of 260/280 absorbance values as well as results from the Agilent Bioanalyzer 2100 confirmed that our extracted RNA lacked DNA contamination. RNA was then amplified at Ambion using the MessageAmp II Biotin kit (Ambion, #1791). First, oligo(dT) primers containing a T7 promoter sequence were introduced to the sample RNA, along with reverse transcriptase and nucleoside triphosphates (NTPs) to create single-stranded cDNA. After this, complementary strands were synthesized in a similar manner with DNA polymerase, and the newly formed double stranded DNA was used as template for RNA amplification using T7 RNA polymerase and biotinylated NTPs. The amplified RNA (aRNA) was fractionated before being introduced to the GeneChip. RNA was allowed to hybridize for 16 hours, after which the array was exposed to streptavidin phycoerythrin conjugate, resulting in fluorescent emissions at 570 nm in proportion to the number of bound aRNA transcripts. The Affymetrix GeneChip Scanner 3000 was used to read emissions levels for each probe space. Results were subsequently quantified as a measure of gene expression. Further details are provided in Research Section 3.

General statistical analyses

DEVELOPMENTAL AND PHYSIOLOGICAL ENDPOINT ANALYSIS

Data from the F1 and F2 generations were analyzed independently due to methodological differences between the two groups. Serial data such as body weight and anogenital distance were analyzed via repeated measures ANOVA using Statview software and the unit of significance was the mean of each litter. Remaining endpoints were analyzed via linear mixed

model ANOVA using SAS software and the unit of significance was an experimental animal. Models using a fixed treatment effect, a random dam (treatment) effect, and a fixed categorical covariate representing day of the estrous cycle at time of euthanasia were fit to the data. Significance was determined by a z-score or F-value for random and fixed terms, respectively. Non-normally distributed data were submitted to a non-parametric permutation test. Tukey-Kramer adjusted post-hoc analyses were performed to determine the source of significance in all instances. These statistics were calculated using SAS software.

BEHAVIORAL ENDPOINT ANALYSIS

Each trait was tested to determine if it was normally distributed. SAS statistical software was used to perform a linear mixed model ANOVA with a fixed treatment effect and a random dam (treatment) effect, and a fixed covariate of the log transform of mating trail length to control for mating time. Statistical significance was determined by a z-score or an F-value for random and fixed terms, respectively. Non-normally-distrbuted traits were log (1+y) transformed, and highly skewed traits were tested with a permutations procedure. Tukey-Kramer adjusted post-hoc t-tests were used to locate the source of significance, when applicable.

MICROARRAY ANALYSIS

The Affymetrix Gene Chip Operating Software (GCOS) v1.3 was used to quantify image signal results. Hybridization quality control measures including scaling factor (a measure of effective labeling), background noise (relates the presence of impurities), and % Present (referring to the % probe sets detectable, related to RNA quality and hybridization success), and 3'/5' ratio of the housekeeping genes GAPDH and actin (a measure of aRNA

integrity) were used to confirm accurate hybridization prior to analysis. Microarray expression values were determined via robust multichip analysis (RMA) (Irizarry, et al., 2003a, Irizarry, et al., 2003b). This probe-level approach normalizes the distribution of PM vs. MM signal intensity values for all probes within a probe set across all arrays. The resulting values were expressed in $\log_2$ format, and underwent batch effect statistics to adjust for any variability between the two microarray runs. Following this, data were submitted to ANOVA, using the Fs statistic. Shuffling biological samples 1000 times generated a permutations-based null hypothesis of no differential expression among treatments. Genes with $p < 0.01$ were listed in tabular form, and 27 neuroendocrine related genes were selected for Tukey-Kramer post-hoc analysis to determine significant between-group interactions. Five genes from this list were chosen for quantitative real time PCR follow-up to be conducted following the publication of this dissertation. Transcriptional response element (TRE) analysis was performed using Genomatix software. Functional groups were determined by gene ontology (GO) classifications in an online version of Multi-Protein Survey System (MPSS) software, and a customized neuroendocrine-specific functional categorization was performed through literature and sequence searches on publicly available databases. Cluster analysis using Adaptive Quality-Based Clustering software was conducted for genes in the list of significant genes ($p < 0.01$) and the resulting 11 clusters were grouped according to dose-response curve shape. Research Section 3 contains additional details on microarray analysis methodology.

Research Section 1:

Prenatal exposure to PCBs in females: development, reproductive physiology, and second generational effects

Abstract

Prenatal exposures to endocrine-disrupting chemicals such as polychlorinated biphenyls (PCBs) can cause latent effects on reproductive function. Here, we tested whether PCBs administered during late pregnancy would compromise reproductive physiology in both the fetally-exposed female offspring (F1 generation), as well as in their female offspring (F2 generation). Pregnant Sprague-Dawley rats were treated with the PCB mixture Aroclor (A) 1221 (0, 0.1, 1 or 10 mg/kg) on embryonic days 16 and 18. Somatic and reproductive development of F1 and their F2 female offspring were monitored, including ages of eye opening, pubertal landmarks, and serum reproductive hormones. The results showed that low doses of A1221 given during this critical period of neuroendocrine development caused differential effects of A1221 on F1 and F2 female rats. No significant differences were observed between treated groups and control in the F1, although the F2 generation showed more profound alterations. In particular, on proestrus, the day of the preovulatory GnRH/LH surge, F2 females exhibited profoundly suppressed LH and progesterone, and correspondingly smaller uterine and ovarian weights on estrus. These latter physiologic changes suggest that the GnRH/LH surge is delayed or abolished in adult F2 female rats whose mothers were fetally exposed to A1221. Thus, low levels of exposure to PCBs during late fetal development cause significant

consequences on the maturation and physiology of two generations of female offspring, and these findings have implications for reproductive health and fertility of wildlife and humans.

Introduction

Polychlorinated biphenyls (PCBs) were used as non-flammable lubricants and insulators in industry beginning in 1929 until they were banned in 1977. Because of their lipophilic structures, PCBs are easily absorbed from the environment into the food chain, rendering human and animal exposure ubiquitous and persistent. As a result, a greater understanding of the many negative outcomes of exposure continues to be essential to human health, and for addressing declining wildlife fecundity and viability.

PCB exposure has been linked with a broad spectrum of effects, both *in viv*o and *in vitro*, which vary depending on method/age of exposure, sex of the individual, and dose/duration of exposure. Fetal and early developmental exposure to PCBs is particularly devastating, and can have different outcomes from adult exposure (Crews, et al., 2000). Latent effects of early exposures include, but are not limited to, depressed circulating thyroid hormone and abnormal thyroid cytology (Bansal, et al., 2005, Chauhan, et al., 2000, Goldey, et al., 1995, Morse, et al., 1996c, Porterfield, 1994), delayed cognitive development (Chen, et al., 1992, Jacobson and Jacobson, 1997), altered sensory and motor abilities (Bowman, et al., 1981, Lasky, et al., 2002, Roegge, et al., 2004), reproductive impairment (Arnold, et al., 1995, Meerts, et al., 2004b, Sager and Girard, 1994, Yang, et al., 2005), and compromised neural function (Donahue, et al., 2004, Morse, et al., 1996a, Provost, et al., 1999a, Seegal, et al., 2005).

Not only do PCBs and other environmental endocrine disrupting chemicals directly affect the exposed individual, but they can also exert effects on subsequent generations that may differ from those associated with primary exposure (Fernie, et al., 2003, Shipp, et al., 1998). One compelling mechanism for multigenerational effects of PCBs is via the hypothalamic-pituitary-gonadal reproductive axis. Exposure of the first generational

animals can later result in aberrant reproductive physiology and behavior, including improper steroid hormone production during pregnancy, and deficiencies in parturition, lactation and maternal behavior (Boersma and Lanting, 2000, Takser, et al., 2005). Such alterations can potentially be transmitted to a second generation offspring through improper hormonal exposure while *in utero*, and altered parental care postnatally (Champagne and Meaney, 2001, Cummings, et al., 2005). Thus PCBs, particularly developmental exposures, can affect the immature organism, adult, and subsequent generations of offspring, although relatively little is known about the nature and mechanisms of these outcomes.

The current study investigated the effects of prenatal exposure to PCBs on sexual and somatic development of two generations, employing low, ecologically relevant doses of PCBs (Zhang, et al., 2004). Aroclor (A)1221, a commercial PCB mixture composed of lightly-chlorinated isomers, was chosen for this study because of its previously reported disruption of the neuroendocrine system (Chung and Clemens, 1999, Chung, et al., 2001, Gore, 2001, Gore, et al., 2002, Salama, et al., 2003, Steinberg, et al., 2007, Woodhouse and Cooke, 2004).

Materials and Methods

ANIMALS AND HUSBANDRY

Sprague-Dawley rats were fed low-phytoestrogen Harlan-Teklad 2019 Global Diet *ad libitum* and were housed in a 12:12 partially reversed light cycle (lights on 2300 h, lights off 1100 h). All animal procedures were conducted in compliance with protocols approved by IACUC at the University of Texas at Austin. One day timed-pregnant Sprague-Dawley rats (F0 generation) were purchased from the Animal Resources Center at the University of Texas at Austin. Dams (n=11-13 per treatment) were injected

i.p. with Aroclor (A)1221 (0.1, 1, or 10 mg/kg; Accustandard #C-221N-50MG; Lot#072-202) or vehicle (0.1 ml DMSO 99.5%; Sigma, #D4540, Lot#122K0027) on gestational days 16 and 18, during the period of embryonic brain sexual differentiation (Ramaley, 1979). Intraperitoneal injection has been used in other studies investigating the effects of PCB exposure (Chung and Clemens, 1999, Gillette, et al., 1987, Murugesan, et al., 2005), and here it was chosen to avoid stress of gavage or possible variability in consumption of infused food pellets.

PCBs are only partially conducted from dam to offspring. To estimate actual exposure of each pup to the dose administered the dam, we followed the methods of Takagi et al, 1986 (Takagi, et al., 1986) and estimated that individual pups in each of the three dosed groups were exposed to approximately 0.2, 2, or 20 μg/kg PCBs, respectively. Because A1221 is a lightly chlorinated PCB mixture, it is believed to be relatively volatile (Thomas, et al., 1998a) with a short half life of several days (Tanabe, et al., 1981), and its low degree of chlorination renders it difficult to detect (Frame, 1997b). We were thus unable to directly confirm body burden of experimental animals following dosage of the pregnant dam. The doses used in this experiment were chosen to approximate human and wildlife exposures. Average PCB serum levels of full term babies in Germany have been measured at 0.5 μg/L (Lackmann, 2002), and from this figure, adipose PCB levels can be estimated at 50 μg/kg (Stellman, et al., 1998). Given these estimates, the doses used in this study closely approximate typical human exposures.

The day of parturition, determined by the birth of at least one pup before lights out at 1100 h, was termed postnatal day (P) 0. On P1, live and dead pups were counted, sex ratio was determined, and litters were culled to 4 females (average litter size = 12) to minimize effects of litter composition

on body size, physiology, and behavior. This cohort is referred to as the F1 generation. At least one adult F1 female per litter was later allowed to become impregnated and produce the F2 generation. F2 litters were culled to 6 females when possible. Although treatment groups were known to the experimenter in the first cohort of F1 and F2 animals (14 litters) for the developmental measures of body weight and anogenital distance, in subsequent cohorts (32 litters) experimenters were blind to treatment for these endpoints. The number of litters per treatment and average numbers of individual per litter are given in Table 2. For all hormonal and postmortem measures, the investigators were blind to treatments for all 46 litters.

Table 2. Numbers of F0, F1, and F2 animals.

Generation:	**F0**				**F1**				**F2**			
A1221 dose (mg/kg) :	**0**	**0.1**	**1**	**10**	**0**	**0.1**	**1**	**10**	**0**	**0.1**	**1**	**10**
Number of dams	13	11	11	11	13	11	11	11				
P1 female					13, 5.46	11, 7.09	10, 7.50	11, 5.36	11, 5.18	8, 7.00	10, 6.40	11, 4.73
P1 male					12, 7.08	11, 6.09	10, 5.30	11, 6.73	11, 6.55	8, 5.38	11, 5.27	11, 6.36

Table 2: The number of litters and the average number of rats per litter (separated by a comma) are shown. For F1 and F2 generations, numbers of litters are shown in the top of each cell, with the average numbers of pups per litter prior to culling shown at the bottom. F1 litters were culled to 4 females and F2 litters to 6 females, on average.

DEVELOPMENTAL MEASURES

Litter size and sex ratios, number of dead pups, anogenital distance (a marker of masculinization/defeminization (Marois, 1968)), and body weight were recorded for F1 and F2 male and female pups on P1, prior to culling. After culling, the remaining females' body weights and anogenital distance were recorded 2-3 times per week until P30 or vaginal opening. Anogenital distance was measured with digital calipers and was independently confirmed by a second investigator. Anogenital distance values were normalized to the cubed root of body weight for analysis (Gallavan, et al., 1999). Age at eye opening was recorded. On P22, pups were weaned to 3-4 littermates per cage. After P30, body weights were recorded once per week, and again on the day of euthanasia. Timing of puberty was quantified as age at vaginal opening (VO), first estrus (FE), and first diestrus (FD; (Salama, et al., 2003)). For both the F1 and F2 generations, daily vaginal smears were conducted from vaginal opening until euthanasia, excepting pregnant or lactating F1 rats.

Intact F1 females were mated (~P60) on the afternoon of proestrus to generate a sperm-positive female for producing the second generation, and in conjunction with another experiment (Steinberg, et al., 2007). Experimental animals were euthanized by unanesthetized decapitation approximately 16 hours later, 1-2 hours before lights out, in random order. F2 experimental animals were subject to the same measures and were euthanized at the same time of day as the F1 generation, although they differed from the first generation in the following three ways: 1) F2 females were sexually naive, 2) they were euthanized at a younger age, on ~P42, and 3) they were randomly cycling at the time of euthanasia with vaginal cytology noted. For both generations, ovaries and uteri were dissected out, and wet weights were recorded. Trunk bloods were collected and

were centrifuged at 6000 x g for five minutes to separate serum. Serum was stored at -80 C until hormone analysis.

SERUM HORMONE MEASUREMENTS

Serum luteinizing hormone (LH) for each animal was assayed in duplicate samples (50 μl) in a single radioimmunoassay (RIA) with LH anti-serum (NIDDK-anti-rLH-11). The iodination preparation is NIDDK-rLH-I–9, iodinated with ^{125}I. The reference preparation used for the standard curve is NIDDK-rLH-RP-3. These were obtained from the National Hormone and Pituitary Program of NIDDK and kindly provided by Dr. A.F. Parlow. The sensitivity of the LH assay was 40 ng/ml at 90% binding, and intrassay CV was 2.36%. Serum estradiol was measured in duplicate (200 μl) samples via the Diagnostic Systems Laboratories DSL-4800 estradiol RIA kit within a series of five runs, according to the kit protocols. Sensitivity is 5 pg/ml. Intra-assay CVs for each of the five estradiol RIA assays were 3.53, 9.54, 4.33, 7.63, 5.42%, respectively, and inter-assay CV was 9.43%. Serum progesterone was measured in duplicate samples of 25 μl via the DSL-3900 progesterone RIA kit, in accordance with kit protocols. Sensitivity is 0.12 ng/ml. Samples were run in a total of 8 assays, for which intra-assay variabilities were 3.89, 1.82, 0.69, 2.76, 8.09, 1.13, 3.06, and 1.52%, respectively, and inter-assay CV was 14.50%.

STATISTICAL ANALYSES

Except where noted, generation 1 and 2 were analyzed independently due to methodological differences between the two experimental generations. Repeated measures ANOVA was used to assess significant differences between treatment groups for serially recorded body weights and anogenital distances. For these endpoints the

unit of statistical analysis was the mean of each litter. The remaining endpoints (ovarian and uterine weight and circulating hormone levels), which exhibited considerably more variability, were analyzed using a linear mixed model ANOVA approach with Proc Mixed in SAS (Cassell, 2002, Littell, et al., 1999, Steinberg, et al., 2007), with consultation and analyses kindly provided by Dr. Thomas Juenger. Here, we fit models that included a fixed treatment effect, a random dam (treatment) effect, and a fixed categorical covariate representing estrous cycle stage of the appropriate category (estrus, diestrus 1, diestrus 2, or proestrus). In addition, our analyses tested for significant interactions between the treatment effect and the estrous cycle stage. If significant interactions were detected, we completed subsequent analyses splitting the data by cycle stage to more fully explore the biological basis of the interaction. Models were estimated by restricted maximum likelihood and significance was determined by a z-score or F-value for random and fixed terms, respectively. Several of the endpoints (LH, estradiol, uterine weight, and body weight) were non-normally distributed and therefore we utilized a non-parametric permutation testing approach to determine significance. Here, phenotypes were randomized with respect to the experimental effects 1000 times and analyses were then carried out as above (Cassell, 2002). The test statistics from the randomized analyses were then used to determine the distribution of the test statistic under the null hypothesis and to empirically obtain at an alpha level of 0.05. Where significant findings were observed, we employed a series of post-hoc t-tests for all combinations of treatments, controlling for multiple tests with a Tukey-Kramer adjustment. LH release is pulsatile, and LH surges occurring on the evening of proestrus combine pulsatile release with rapidly escalating baseline levels. Collecting blood at a single time point makes detection of outliers difficult;

therefore, suspected outliers were tested with Grubb's outliers test (Iglewicz and Hoaglin, 1993),, and confirmed outliers were eliminated from statistical analysis.

Results

F1 LITTER COMPOSITION ON P1

A1221 did not affect litter sizes or sex ratios in the F1 generation (Figure 3A). Post-hoc analysis revealed no specific significant interactions between treatment groups, although the control vs. 0.1 mg/kg group had a p-value of $p < 0.09$ in F2 sex ratio, suggesting a trend for differences between these groups.

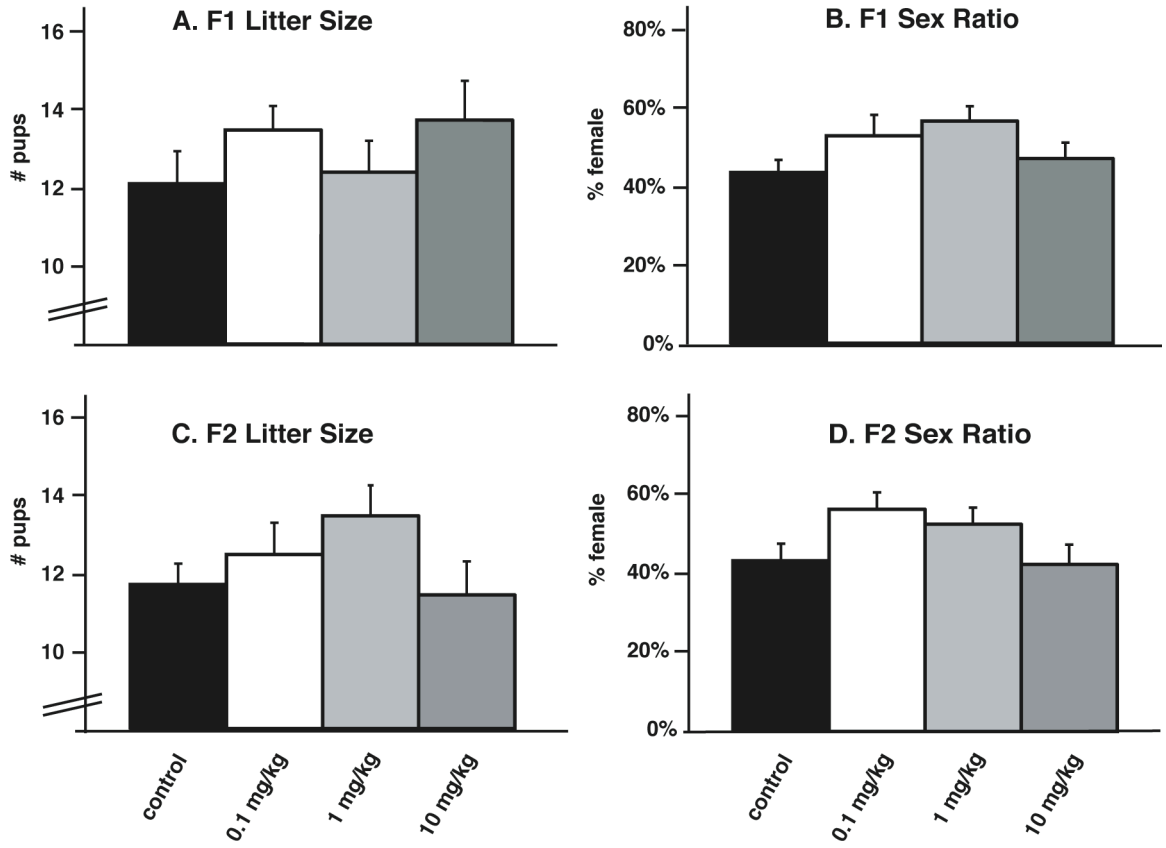


Figure 3: F1 & F2 litter size and litter sex ratios. A. Numbers of pups per litter (prior to culling) in the F1 generation group did not differ with treatment. B. Sex ratios did not differ in either the F1 or the F2.

F1 BODY WEIGHT AND ANOGENITAL DISTANCE ON P1

Body weight and anogenital distance were measured for P1 male and female pups, as shown in Table 3. A1221 did not affect anogenital distance in male pups on P1, nor did it affect birth weight in pups of either sex (Table 3).

Table 3. Body weight and anogenital distance of F1 pups on P1.

	Control	**0.1 mg/kg A1221**	**1 mg/kg A1221**	**10 mg/kg A1221**
P1 female body weight (g)	6.10 ± 0.19 (n=71)	5.98 ± 0.22 (n=78)	6.32 ± 0.18 (n=76)	5.94 ± 0.18 (n=59)
P1 female anogenital ((mm/g^3)	0.74 ± 0.03 (n=71)	0.81 ± 0.02[a] (n=78)	0.71 ± 0.06 (n=76)	0.73 ± 0.03 (n=59)
P1 male body weight (g)	6.36 ± 0.21 (n=85)	6.26 ± 0.24 (n=67)	6.52 ± 0.25 (n=53)	6.33 ± 0.18 (n=74)
P1 male anogenital distance (mm/g3)	1.53 ± 0.04 (n=85)	1.58 ± 0.03 (n=67)	1.59 ± 0.06 (n=53)	1.62 ± 0.07 (n=74)

Body weights and anogenital distance were measured on P1, prior to culling litters. Anogenital distances are shown normalized to body weight. All data are shown as mean ± SEM. Number of individual animals per treatment (n) is given .Female anogenital distance: The 0.1 mg/kg group female anogenital distance (normalized to body weight) tended to be larger than other groups ($p = 0.054$ vs. 1 mg/kg; $0.05 < p < 0.1$ vs. 10 mg/kg). Female body weight: No significant differences were found on P1. Male anogenital distance, body weight: No significant differences were detected.

F1 BODY WEIGHT AND ANOGENITAL DISTANCE DURING DEVELOPMENT

After litters were culled, body weights and anogenital distance were recorded over time for each remaining experimental female (Figure 4). A repeated measures 2-way ANOVA examining the effects of age and treatment on body weight was performed for F1 females, revealing a significant effect for treatment ($p < 0.01$) and a treatment x age interaction ($p < 0.0001$). *Post-hoc* analysis revealed that interactions were attributable to P34. On P34, the 1 mg/kg group was significantly heavier than all other groups ($p < 0.005$ for all).

Anogenital distance was also affected in the F1 females (Figure 4). Although treatment alone did not cause any effects ($p = 0.126$), age was associated with significant differences in anogenital distance ($p < 0.0001$). A trend for an interaction of age with treatment was also found ($p = 0.056$). Overall, the 0.1 mg/kg group had the largest anogenital distance, and the 10 mg/kg A1221 group had the smallest anogenital distance. *Post-hoc* analysis confirmed differences between groups on P9 and P13, when the 0.1 mg/kg group had a significantly larger anogenital distance than either the control or 10 mg/kg group ($p < 0.05$ for both). Anogenital distances were slightly but non-significantly different on P16 and P23, with a trend for a larger anogenital distance detected in the 0.1 mg/kg group and a smaller anogenital distance in the 10 mg/kg group.

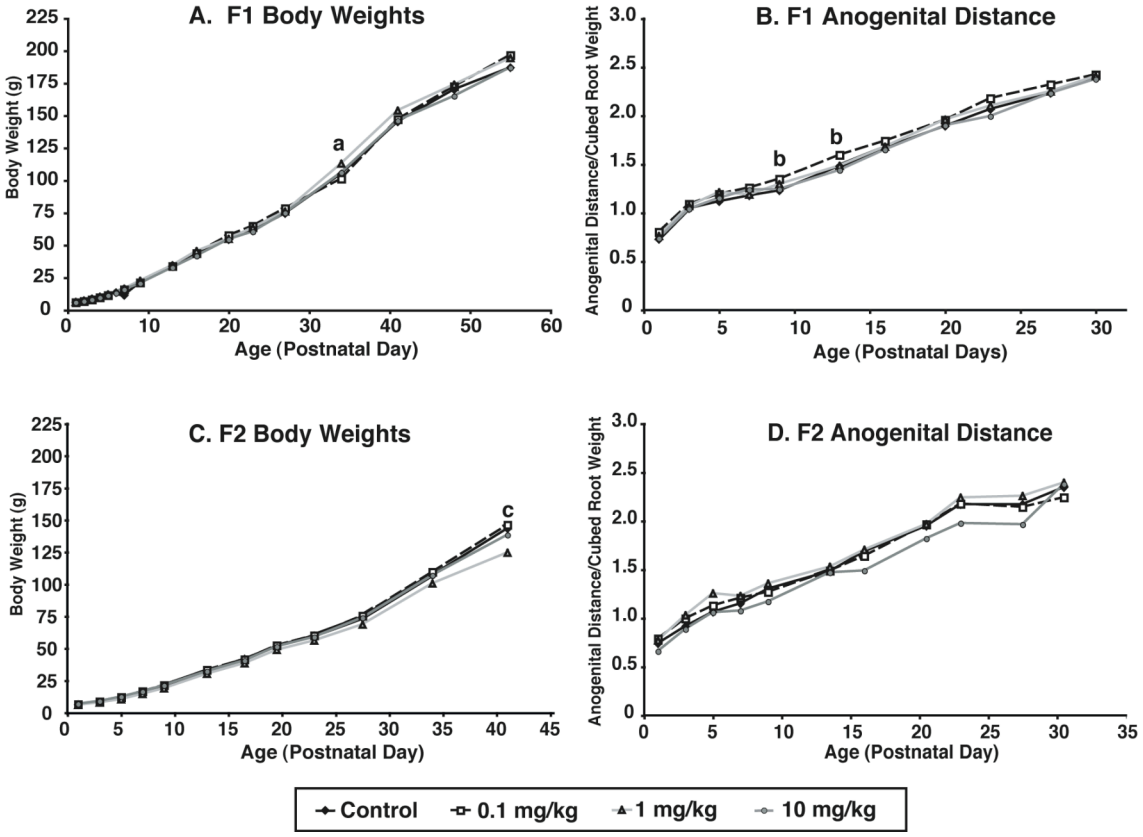


Figure 4: F1 and F2 body weight and anogenital distance. Body weights and anogenital distances were analyzed by repeated measures ANOVA across development. Number of individuals in the control, 0.1, 1, and 10 mg/kg groups were, respectively: 55, 43, 47, and 40 for the F1, and 50, 51, 41, and 47 for the F2. These numbers were the same for timing of developmental events (Table 4). A. F1 body weights: On P34, the 1 mg/kg group was significantly heavier than all other treatment groups ($p < 0.005$ for all). B. F1 anogenital distance: *Post-hoc* analysis identified differences on P9 (0.1 mg/kg vs. control and 10 mg/kg, $p < 0.05$), and P13 (0.1 mg/kg vs. control and 10 mg/kg, $p < 0.05$), C. F2 body weights: On P40-42, the 1 mg/kg group was significantly lighter than the control or 0.1 mg/kg groups ($p < 0.05$). D. F2 Anogenital Distance was unaffected by treatment. a. 1 mg/kg vs. all other treatments ($p < 0.005$), b. 0.1 mg/kg vs. control and 10 mg/kg ($p < 0.05$), c. 1 mg/kg vs. control and 0.1 mg/kg ($p < 0.05$).

F1 POSTNATAL MATURATION

As shown in Table 4, there were no significant effects of A1221 treatment on the developmental markers of eye opening, vaginal opening, first estrus, and first diestrus.

Table 4. F1 postnatal maturational markers.

	Control	0.1 mg/kg A1221	1 mg/kg A1221	10 mg/kg A1221
Eye Opening	14.9 ± 0.1	14.8 ± 0.1	15.1 ± 0.1	15.1 ± 0.1
Vaginal Opening	33.4 ± 0.4	33.4 ± 0.4	33.6 ± 0.3	33.5 ± 0.5
First Estrus	34.6 ± 0.7	34.4 ± 0.7	34.3 ± 0.5	33.7 ± 0.6
First Diestrus	35.6 ± 0.4	35.3 ± 0.5	36.0 ± 0.5	35.7 ± 0.4

Data shown are in postnatal age in days, mean ± SEM. No significant differences were observed between groups for any postnatal maturational markers.

F1 SERUM HORMONE LEVELS

Trunk blood samples collected ~20 hours post-mating were used for measurement of circulating luteinizing hormone (LH), progesterone, and estradiol levels. No significant effects of A1221 treatment were detected for serum progesterone and estradiol (Figure 5A and 5B, respectively. However, serum LH concentrations were significantly altered ($p < 0.05$; Figure 5C). *Post-hoc* analyses showed that the 1 mg/kg group was significantly different from the 0.1 and 10 mg/kg groups ($p < 0.05$) and tended to be different from the control group ($p = 0.0516$). Rat estrous cyclicity was monitored by daily vaginal smears beginning at VO and no apparent differences were detected (data not shown).

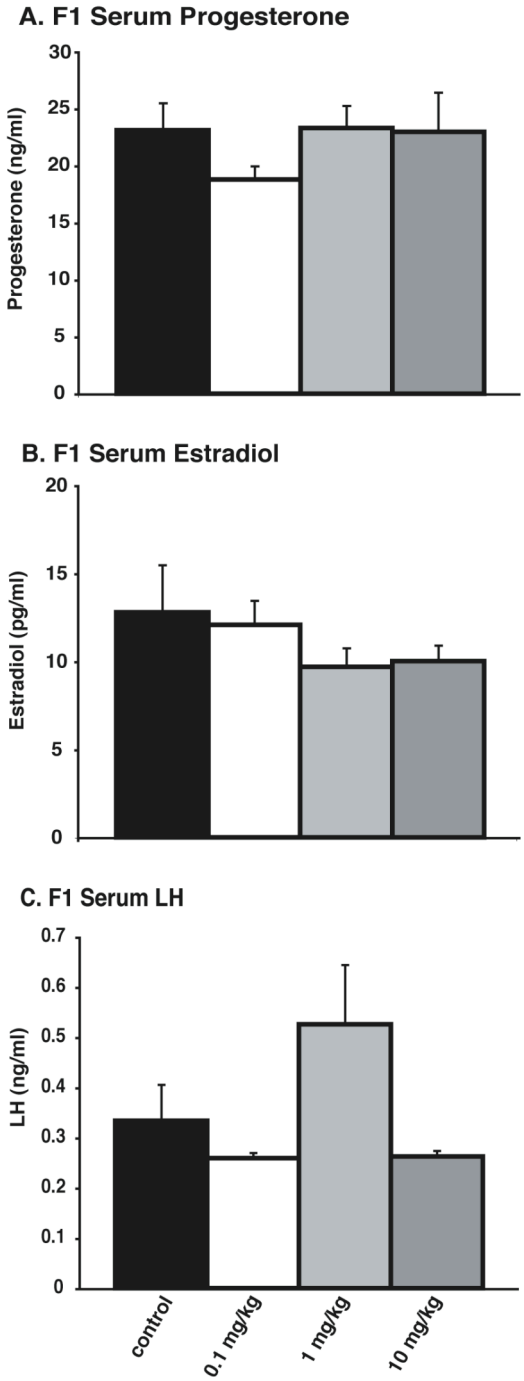


Figure 5: F1 serum hormone levels. A. Serum progesterone; number of individuals in control, 0.1, 1, and 10 mg/kg groups were 20, 22, 17, and 17, respectively. B. Serum Estradiol; number of individuals in control, 0.1, 1, and 10 mg/kg groups were 19, 20 15, and 15, respectively. C. Serum luteinizing hormone (LH); number of individuals in control, 0.1, 1, and 10 mg/kg groups were 19, 20, 17, and 16, respectively. No significant differences were observed for progesterone and estradiol. Although the 1 mg/kg group was found to have significantly higher LH levels than either the 0.1 or 10 mg/kg groups ($p < 0.05$), the comparison with control was only slightly higher than the 0.05 significance cutoff after post-hoc adjustment ($p = 0.0516$). Data are shown as mean $\pm$ SEM.

F1 OVARIAN/UTERINE WEIGHTS

The uterus and paired ovaries were carefully dissected and weighed after euthanasia. There was no effect of A1221 treatment on F1 uterine or ovarian weights, shown normalized to body weight (Figure 6).

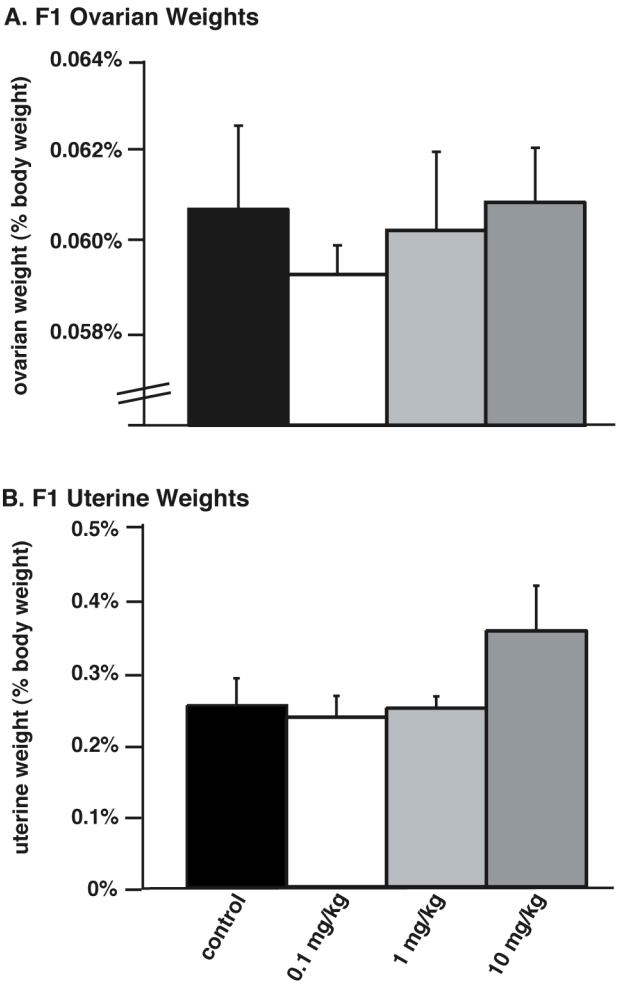


Figure 6: F1 ovarian and uterine weights, shown normalized to body weight. There were no significant differences between groups. Number of individuals per group for control, 0.1, 1, and 10 mg/kg groups were 36, 31, 34, and 29, respectively. Data are shown as mean ± SEM.

F2 LITTER SIZE AND COMPOSITION ON P1

Litter sizes of the F2 generation are shown in Figure 3. No significant differences were found. For sex ratio, as with the first generation, no significant effects were observed.

F2 BODY WEIGHT AND ANOGENITAL DISTANCE ON P1

On the day after birth, body weights in F2 generation females were smallest in the 1 mg/kg A1221 group although this was only significantly different from the 10 mg/kg group which had the highest mean body weight ($p < 0.05$; Table 5). Several significant differences were found for body weight in F2 male rats on P1. Like the females, the 1 mg/kg group had lower body weights and the 10mg/kg group had the highest body weights (1 mg/kg vs. 10 mg/kg: $p < 0.01$). Although not significantly different from controls, a trend was found for differences in body weight between 1 mg/kg and control pups. The male control group pups were also significantly smaller than the 10 mg/kg group ($p < 0.01$; Table 5). Anogenital distances were equivalent across all groups for males and for females (Table 5).

Table 5. Body weight and anogenital distance of F2 pups on P1.

	Control	**0.1 mg/kg A1221**	**1 mg/kg A1221**	**10 mg/kg A1221**
P1 female body weight (g)	6.22 ± 0.22 (n=57)	6.53 ± 0.26 (n=64)	5.89 ± 0.23[c] (n=64)	6.68 ± 0.16 (n=52)
P1 female anogenital distance (mm/g^3)	0.75 ± 0.04 (n=50)	0.79 ± 0.04 (n=64)	0.77 ± 0.04 (n=64)	0.72 ± 0.02 (n=52)
P1 male body weight (g)	6.61 ± 0.15[a] (n=72)	6.89 ± 0.32 (n=50)	6.16 ± 0.20 (n=58)	7.04 ± 0.15[a] (n=73)
P1 male anogenital distance (mm/g^3)	1.57 ± 0.05 (n=72)	1.64 ± 0.06 (n=50)	1.60 ± 0.10 (n=58)	1.62 ± 0.06 (n=73)

Body weights and anogenital distance were measured on P1, prior to culling litters. Anogenital distances are shown normalized to body weight. Number of individuals per treatment per endpoint are given in parentheses. Data are shown as mean ± SEM. Female anogenital distance: No differences were detected. Female body weight: The 1 mg/kg group had smaller body weights than the 10 mg/kg group ($p < 0.05$), but did not significantly differ from control. Male anogenital distance: No significant differences were found. Male body weight: The control group was significantly smaller than the 10 mg/kg group ($p < 0.01$). The 1 mg/kg group was also significantly smaller than the 10 mg/kg group ($p < 0.01$). a. $p < 0.01$

F2 BODY WEIGHT AND ANOGENITAL DISTANCE DURING DEVELOPMENT

Repeated measures ANOVA showed that F2 female body weights were significantly affected by age ($p < 0.0001$) but not A1221 treatment ($p = 0.28$), although a significant interaction of age with treatment was detected ($p < 0.02$; Figure 4). In general, body weights were smallest in the 1 mg/kg group, and largest in the 10 mg/kg group. *Post-hoc* analysis showed significantly larger body weights of the 10 mg/kg group vs. the 1 mg/kg group on P1 and 3 ($p < 0.05$), and P5 ($p < 0.01$). On P5, the 1 mg/kg group also tended to be heavier than the 0.1 mg/kg group ($p < 0.07$). However, on P40-42, the 1 mg/kg group was significantly lighter than the control or 0.1 mg/kg groups ($p < 0.05$). F2 female anogenital distance was similar across all groups as no main or interaction effects were detectable via repeated measures ANOVA (Figure 4).

F2 POSTNATAL MATURATIONAL MARKERS

There were no significant differences between treatment groups for developmental markers of EO, VO, FE, or FD (Table 6).

Table 6. F2 postnatal maturational markers.

	Control	0.1 mg/kg A1221	1 mg/kg A1221	10 mg/kg A1221
Eye Opening	15.2 ± 0.2	15.2 ± 0.2	15.3 ± 0.2	15.2 ± 0.2
Vaginal Opening	33.2 ± 0.3	33.5 ± 0.3	33.4 ± 0.6	33.0 ± 0.4
First Estrus	33.6 ± 0.4	33.8 ± 0.3	34.0 ± 0.7	33.8 ± 0.5
First Diestrus	35.3 ± 0.3	35.5 ± 0.3	35.0 ± 0.8	35.9 ± 0.7

Data shown are in postnatal age in days, mean $\pm$ SEM. No significant differences were observed between groups for any postnatal maturational markers.

F2 SERUM HORMONE CONCENTRATIONS

Serum samples were used for assays of progesterone, estradiol and LH concentrations (Figure 7). Uterine/ovarian wet weights were measured (Figure 8). Because rats were randomly cycling, data are presented according to different days of the estrous cycle, across which serum hormone and gonadal/uterine weights exhibit natural fluctuations.

Progesterone: Circulating progesterone concentrations were analyzed on each of the 4 estrous cycle stages: proestrus, estrus, diestrus 1 and diestrus 2. Analyses of interactions between cycle stage and treatment showed significant effects of treatment for rats that were euthanized on proestrus. At that cycle stage, the control group had significantly higher progesterone concentrations than the 0.1 mg/kg group ($p < 0.005$). Control rats also had higher progesterone concentrations than the 1 and 10 mg/kg A1221 groups ($p < 0.05$). On diestrus 2, significant differences were found for the 0.1 and 1 mg/kg groups vs. the 10 mg/kg group ($p < 0.05$).

The treatment x cycle stage interaction was further investigated via post-hoc analysis to detect differences between circulating progesterone levels across the estrous cycle within each treatment group. All treatments showed significant interaction between estrous cycle stage and treatment: the control and 1 mg/kg groups at $p < 0.05$, and the 0.1 and 10 mg/kg groups at $p < 0.001$. Tukey-Kramer adjusted post-hocs for the control group revealed significant differences between progesterone levels on estrus versus diestrus 1 ($p < 0.01$) and between diestrus 1 and proestrus ($p < 0.05$). In comparison, of the PCB treated groups, only the 10 mg/kg group exhibited significantly different progesterone levels between estrus and diestrus 1 ($p < 0.001$), and only the 0.1 mg/kg group showed significant differences between diestrus 1 and proestrus ($p < 0.01$). In addition, all three treatment groups

had significantly lower progesterone levels on proestrus versus estrus, unlike control ($p < 0.01$ for the 0.1 mg/kg group and $p < 0.001$ for the 1 and 10 mg/kg groups). The 10 mg/kg group also exhibited differences between estrus and diestrus 2 ($p < 0.001$).

Estradiol: While a significant effect of cycle stage ($p < 0.001$) was detected by two-way ANOVA, no effect of treatment ($p = 0.36$), nor any interaction of treatment with cycle stage ($p = 0.13$) was detected.

LH: LH levels were non-normally distributed, probably due to pulsatile release and the presence/absence of the LH surge, thus non-parametric permutations statistics were used. LH levels varied significantly by estrous cycle stage ($p = 0.01$). A significant interaction of treatment with cycle stage was also found ($p < 0.05$). Significant interactions or trends were found on proestrus for control vs. 0.01 mg/kg ($p < 0.01$), 1 mg/kg ($p < 0.05$) and 10 mg/kg ($p < 0.01$). Because a significant treatment x estrous stage effect was observed, post-hoc analyses were performed on the interactions to determine possible differences between LH levels across the estrous cycle of each treatment group. Only the control and 10 mg/kg groups showed significantly different LH levels across the cycle (control $p < 0.05$, 10 mg/kg $p < 0.01$), suggesting that the 0.1 and 1 mg/kg groups did not vary in LH levels at any point of the estrous cycle tested. Control animals had significantly higher LH levels on proestrus than on diestrus 1 or 2 ($p < 0.05$ for each). The 10 mg/kg group shared the same pattern ($p < 0.01$) and additionally had significantly higher LH levels on proestrus than on estrus ($p < 0.01$).

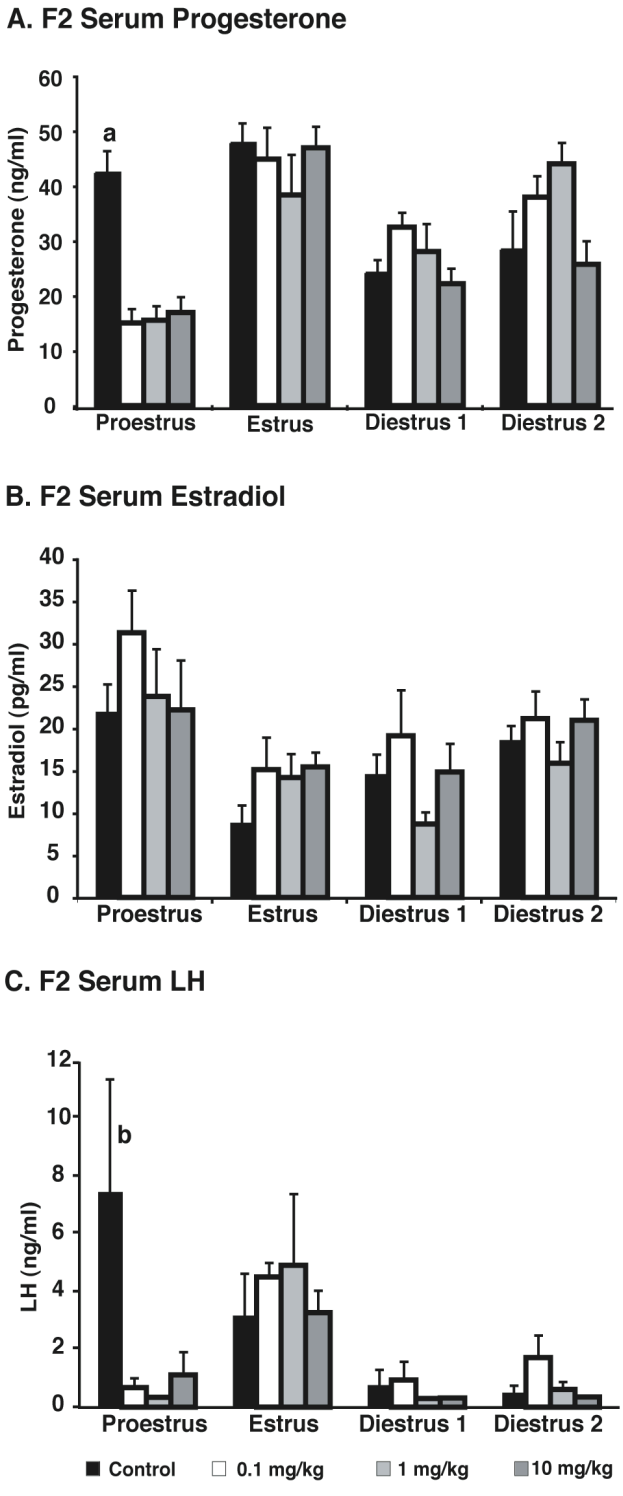


Figure 7: F2 serum hormone levels across the estrous cycle. A. Serum progesterone: Significant differences in serum progesterone levels were found on proestrus ($p < 0.005$ for control vs. 0.1 mg/kg and $p < 0.05$ for control vs. 1 and 10 mg/kg groups) and are designated by the letter a. in the Figure. Number of individuals in control, 0.1, 1, and 10 mg/kg groups were : 34, 51, 34, and 45, respectively. B. Serum Estradiol: a significant effect of cycle stage ($p < 0.001$) was detected, but post-hoc analysis did not detect any specific significant differences between groups. Number of individuals in control, 0.1, 1, and 10 mg/kg groups were 31, 47, 30, and 42, respectively: C. Serum LH: Significant interactions were found on proestrus for control vs. 0.1 mg/kg and 10 mg/kg groups ($p < 0.01$), and control vs. 1 mg/kg group ($p < 0.05$), designated by the letter b. on the figure. Number of individuals with LH data in control, 0.1, 1, and 10 mg/kg groups were : 36, 51, 30, and 47, respectively. Data are shown as mean $\pm$ SEM.

F2 OVARIAN AND UTERINE WEIGHTS

Ovarian and uterine wet weights were measured in randomly cycling rats on ~P42, and results herein are described as a percentage of body weight and presented across the estrous cycle in Figure 8. For the ovary, two-way ANOVA showed a significant effect of treatment ($p < 0.05$) and cycle stage ($p < 0.0001$), but no interaction. *Post-hoc* analyses of treatment showed differences for control vs. 0.1 mg/kg ($p < 0.05$), and a non-significant trend between control vs. 1 mg/kg ($p = 0.051$). Two-way ANOVA for the uterus showed a non-significant treatment effect, but a significant effect of estrous stage while no interaction with treatment.

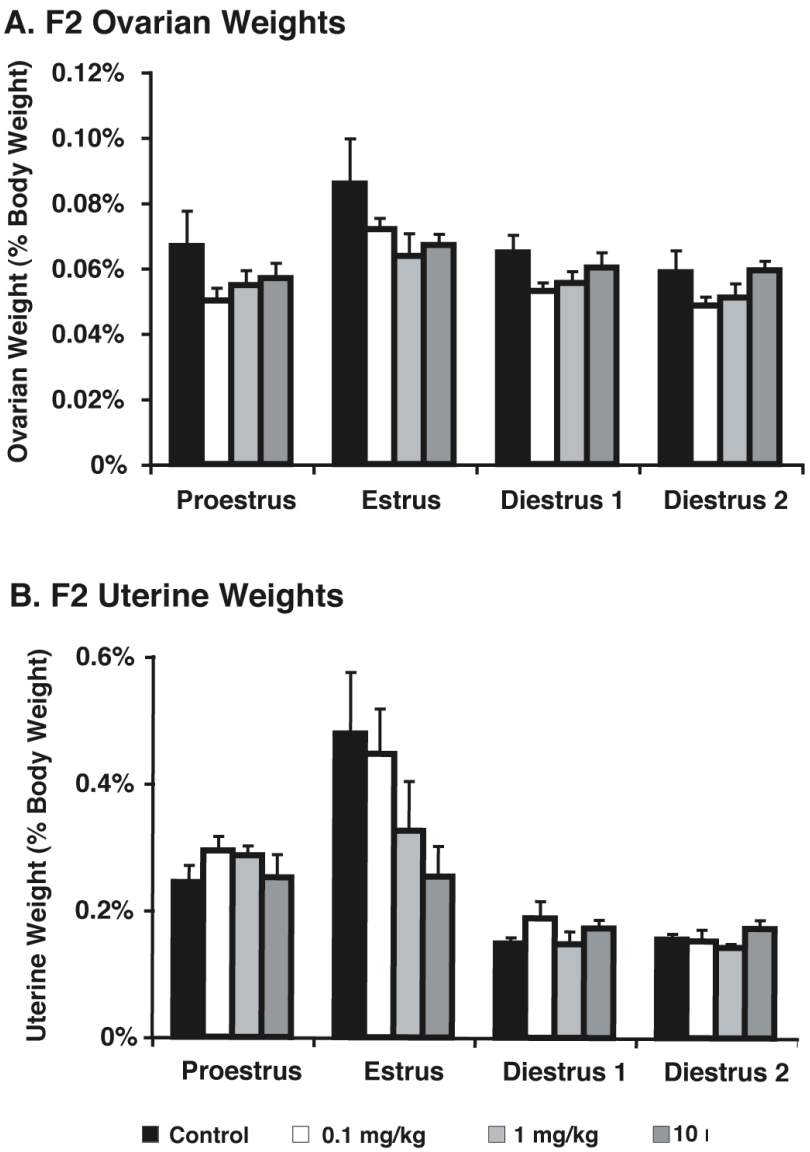


Figure 8: F2 0varian and uterine weights on ~P42, expressed as a percentage of body weight and shown here according to estrous cycle day. The number of individuals per per treatment group for control, 0.1, 1, and 10 mg/kg were, respectively, 38, 51, 34, and 48. Data shown are mean ± SEM. A. Ovarian Weights. Although no interaction was observed between treatment and estrous cycle day, A1221 treatment was found to have a significant overall effect on the control vs. 0.1 mg/kg group ($p < 0.05$). The control vs. 1 mg/kg group comparison also demonstrated a non-significant trend for a difference ($p = 0.0514$). B. Uterine Weights. No significance was observed for uterine weights in the F2 generation.

Discussion

The current study was designed to test the effects of exposures to PCBs on female development, reproductive physiology, and fertility. Although aspects of such work have previously been undertaken, our study brings together three important features. First, we utilized low-dose, environmentally-relevant concentrations of PCBs (Zhang, et al., 2004), and employed a dose-response approach. The latter is particularly important because hormonally-active compounds can act via non-traditional mechanisms such as U- or inverted-U-shaped dose-response curves, and it is important to span a range of dosages to reveal these dose-response relationships (Calabrese and Baldwin, 2001a, De Kloet, et al., 1998, Gore, 2001). Second, we examined effects of fetal exposure of the F1 individuals on adult endpoints. The model of fetal basis of adult phenotype is also important because the developing organism is particularly vulnerable to exposure during periods when organ and neural systems are being organized (Gore, et al., 2006a). Third, our experiments extended analyses to the F2 offspring, in order to assess possible transgenerational effects, something that has been reported for several species (Fernie, et al., 2001, Kaneko, et al., 1974, Mccoy, et al., 1995) and with other environmental toxicants (Anway, et al., 2005).

Direct comparison of the two generations examined within this study was precluded by methodological differences in the experimental protocol applied to each cohort. For example, the first generation was culled to 4 females, while the second was culled to 6, which could promote larger body size in the first generation due to fewer demands on resource allocation. Additionally, circulating hormone profiles and uterine and ovarian weight were likely to be affected by the advanced age and sexual experience

(impregnation) of rats in the first generation, compared to rats in the second generation.

Several indirect mechanisms may also be a factor for differential effects in the first versus second generation. Gestational delivery of PCBs can affect both the dam as well as the embryos. Direct dosing can alter maternal response of the F0 generation dams to their pups (Simmons, et al., 2005) whereas *in utero* PCB exposure of the pups can influence F0 dam maternal behaviors via an unknown signal (Cummings, et al., 2005), with implications for decreased milk let-down (Cummings, et al., 2005). Certain PCBs induce hypothyroidism (Gauger, et al., 2004), which can antagonize lactation and decrease milk quality (Hapon, et al., 2007). If PCB treated F0 dams experienced altered thyroid hormone levels, they may have produced less lactate of a poorer quality, resulting in differential nutritional supply to pups in the first versus the second generation. Finally, developmental exposure of mice to a similar endocrine disrupting chemical, bisphenol A, causes latent deficits in maternal behaviors in adulthood (Palanza, et al., 2002), suggesting that in utero exposure to PCBs may alter adult maternal behaviors of the F1 generation. Abnormal maternal behaviors or lactation may affect the physical development (Smart, et al., 1983) and adult behavioral phenotype (Meaney, 2001) of the offspring .

During statistical analyses, the effect of dam identity, i.e. “dam effects”, on each endpoint were investigated. When significant, dam effects was included as a covariate for analysis; however, it is additionally informative to note which endpoints exhibit dam effects as a clue to understanding how PCB toxicity may be conducted transgenerationally. For example, the effect of dam on uterine weight in the F1 ($p < 0.0001$) and the F2 ($p < 0.0000$) generation was highly significant. This suggests that either the physiology of the F0 dam or the accuracy or exact location of the PCB

injection may have had a large enough impact as to set uterine weight values for the subsequent two generations. There was no interaction between dam effects and ovarian weight in the F1, but this association was highly significant in the F2 ($p > 0.0009$) generation. The lack of association in the F1 is likely due to the fact that ovaries were only harvested on the day of estrus, and this single time point may not be as informative as data representing all days of the estrous cycle, such as in the F2.

Dam effects in the F1 and F2 were associated with different circulating hormones. Whereas dam effects predicted only progesterone levels in F1 animals ($p < 0.01$), dam effects were only linked to estradiol levels in the F2 ($p < 0.05$). It is likely, again that recording hormone levels across the estrous cycle would give more power to F1 analyses to more accurately determine dam effects. Alternatively, the data as presented allow for the possibility that F0 and F1 gestational physiologies affected the hormone state of their offspring in different manners; for example, A1221 may have acted as an estrogen in developing F1 females, resulting in abnormal patterning of the neuroendocrine brain with implications for altered release of progesterone, estrogen, or oxytocin during their pregnancy, This might result in an F2 generation that experienced a different, but equally detrimental, pathological hormonal environment during development, with different implications for adult reproductive physiology.

There were no consistent trends in negative health effects of PCB-exposed females, although one F1 female in the 1 mg/kg treatment group developed mammary tumors of unknown origin during her pregnancy, at approximately 4 months of age. Autopsy found metastasis of the cancer to lungs, uterus, and ovaries, although microscopic histological analysis was not conducted. In addition, one F2 litter in the 10 mg/kg group presented chronic eye irritation characterized by discharge and loss of fur surrounding

the eyes. This was not believed attributable to PCB treatment and no viruses were detected in afflicted individuals.

EFFECTS OF PCBS ON LITTER SIZE AND SEX RATIO IN TWO GENERATIONS (F1 AND F2)

For the current study, we chose dosages of A1221 that mimic environmental exposures (Zhang, et al., 2004), anticipating that these low-level exposures would not have gross morphological effects. Rather, we sought to investigate more subtle and long-lasting outcomes of A1221 treatment. As predicted, A1221 had no significant effect on litter size in the fetally exposed F1 litters.

EFFECTS OF PCBS ON BODY WEIGHT, ANOGENITAL DISTANCE, AND SOMATIC AND SEXUAL DEVELOPMENT ACROSS TWO GENERATIONS (F1 AND F2)

We monitored body weights and measured the anogenital distance of all male and female pups on postnatal day 1 (P1). Body weight is a useful index of maternal lactational physiology or nursing behavior, and can be an indicator of sickly pups. Previous PCB studies have shown decreased birth weight and growth rate in exposed animals (Bowers, et al., 2004, Hany, et al., 1999, Turk and Hietman, 1976), and humans (Taylor, et al., 1984). Nevertheless, in our study, no effects of prenatal A1221 were found for F1 pups on P1. Differences in body weight were detected for the F2 generation. Specifically, body weight in the 1 mg/kg F2 offspring was smallest in both male and female pups. However, we believe this is probably attributable to the larger litter sizes in that group (see Figure 3).

Anogenital distance in the neonate is determined by prenatal androgens (Gray, et al., 1994), and can be a sensitive measure of endocrine

disruption. In the F1 generation, anogenital distances were only different for one group: in the 0.1 mg/kg female F1 pups on P1, anogenital distance was larger than that in all other groups, suggesting a slight masculinization of females. F2 rats did not differ by treatment for anogenital distance.

After P1, the two generations of female rats were also monitored regularly for body weight and anogenital distance throughout the study. For the F1, only one age showed significant differences between a treated and the control group: P34 for the 1 mg/kg treatment group. In the F2 generation, the 1 mg/kg group had the lightest body weight at age P42, just prior to euthanasia, and future research will investigate whether consistently lower body weights in the F2 are associated with the 1 mg/kg treatment level through later time points. The 1 mg/kg treatment group demonstrated the greatest number of disrupted endpoints in a related experiment testing the effects of A1221 on female sexual behaviors (Steinberg, Juenger, and Gore, 2007; in press), and thus it may be significant that this difference in weight occurs during the pubertal period of female rats. No effects on F2 anogenital distance were found.

Despite some differences in body weight among A1221-exposed F1 rats and their F2 offspring, the developmental landmarks of eye opening, vaginal opening, first estrus, and first diestrus were unaffected by PCB exposure. This result is not surprising considering the relatively low doses employed in the current study and is consistent with other reports (e.g. (Salama, et al., 2003)).

EFFECTS OF PCBS ON SERUM HORMONES AND UTERINE/OVARIAN WEIGHT IN TWO GENERATIONS (F1 AND F2)

Serum hormone and gonadal weights were measured in the F1 and F2 generations after euthanasia. Results for the two generations are

discussed separately because of experimental differences: first, F1 females were euthanized the day after mating whereas the F2 generation were euthanized one week post-puberty, were sexually naïve, and were randomly cycling. Second, F2 rats were slightly younger than F1 rats at the time of euthanasia.

In the F1 generation, two of the three hormones measured (progesterone, estradiol) were unaffected by A1221, whereas serum LH concentrations were significantly altered by A1221, specifically in the 1 mg/kg group. This effect of the intermediate PCB dose is similar to other reports on inverted U-shaped dose-response curves (Calabrese and Baldwin, 2003). We did not detect significant differences in ovarian or uterine weights among the four treatment groups. Because most serum hormones and organ weights were unaffected by treatment, differences in the F2 generation discussed below are likely attributable to other mechanisms, such as altered maternal behaviors of F1 rats or altered pregnancy or parturition-dependent phenotypes. Although it was beyond the scope of the present study to quantify maternal behavior, this is a subject of future work.

In the F2 generation, serum hormone data were profoundly altered by A1221. As a whole, the hormonal profiles of progesterone and LH across the estrous cycle suggest that the PCB groups lack a preovulatory gonadotropin surge, or that the surge was abnormally timed. On proestrus, the day of the surge, none of the three treatment groups exhibited the increased progesterone and LH concentrations characteristic of a surge, such as was observed in our control rats. In fact, when considered across the estrous cycle, the hormone profiles are consistent with a dampening and/or a shift in the timing and pattern of circulating hormone levels, an effect that could greatly interfere with fertility. Moreover, low levels of LH on proestrus in the absence of altered estradiol concentrations suggests a difference in the

positive feedback effects of estradiol on that day and are supportive of a hypothalamic and/or pituitary impairment. The results on ovarian and uterine wet weights across the estrous cycle are consistent with our hormonal profiles. Both ovarian and uterine weights were higher on estrus than on other days of the cycle for all groups, but effects were dampened in the PCB groups compared to the control groups. Again, this suggests a decreased ability of these tissues to respond to regulatory hormone signaling, particularly on proestrus and estrus when differences were greatest. As a whole, sex hormone levels and reproductive tract weights of F2 rats indicate a transgenerational effect of A1221 on ovulatory processes. Although we do not know if these same mechanisms were in play in the F1 generation as all rats were euthanized the day after mating, we do know that that generation was fertile and capable of producing the F2 generation. However, we found in other experiments that the F1 PCB-treated rats required additional paced mating trials to mate (Steinberg, et al., 2005) suggesting an uncoupling of reproductive physiology and behavior. In future studies we will characterize the full reproductive ovulatory profiles of F1 rats; however that was beyond the scope of the current study.

POSSIBLE MECHANISMS FOR EFFECTS OF A1221 ON F1 AND F2 FEMALE RATS

The observed *in vivo* effects of PCBs, as well as most other endocrine-disrupting chemicals, are likely attributable to complex actions on multiple systems. For example, PCBs can alter any or all of the following: activity of enzymes involved in hormone or neurotransmitter metabolism or biosynthesis (Drenth, et al., 1998, Kester, et al., 2000, Liu, et al., 1995), mRNA stability (Lin, et al., 2006b, Pocar, et al., 2001), cell and mitochondrial membrane integrity (Tan, et al., 2003, Tan, et al., 2004b), serum hormone

binding proteins (Chauhan, et al., 2000, Hodgert Jury, et al., 2000), along with direct actions on nuclear receptors including the estrogen receptor, thyroid receptor, and aryl hydrocarbon receptor (Kafafi, et al., 1993, Moore, et al., 1997, Schrader and Cooke, 2003). Furthermore, neurotransmitter systems such as norepinephrine (Seegal, et al., 1985), dopamine (Seegal, et al., 1990), serotonin (Morse, et al., 1996b) and acetylcholine (Provost, et al., 1999a) are also targets of PCBs. With particular reference to A1221, weak estrogenic effects (Shekhar, et al., 1997), weak anti-androgenic effects (Schrader and Cooke, 2003), but little or no association with aryl hydrocarbon receptor (Kafafi, et al., 1993) have been reported. For neuroendocrine systems, our laboratory reported that A1221 given perinatally can down-regulate estrogen receptor beta expression in the anteroventral periventricular nucleus (Salama, et al., 2003), a region implicated in female ovulatory function (Gu and Simerly, 1997). In a gonadotropin-releasing hormone (GnRH) GT1-7 cell line, A1221 alters GnRH gene expression (Gore, et al., 2002) and preliminary work from our lab showed a similar effect *in vivo* (Gore, 2001) suggesting that hypothalamic GnRH neurons may be targets of A1221. Finally, A1221 alters adult female sexual behaviors in rat (Chung and Clemens, 1999, Chung, et al., 2001), suggesting a link between effects of A1221 on brain physiology and reproductive behavior.

It is not surprising that the current transgenerational effects of A1221 were observed. Transgenerational effects have been observed in studies of diethylstilbestrol (DES), a transplacental carcinogen and endocrine disrupting chemical previously prescribed to pregnant women. DES leads to tumorogenesis (Walker and Haven, 1997) and decreased fertility (Newbold, et al., 1998, Turusov, et al., 1992) in the F1 offspring of exposed pregnant mice, and can increase tumorogenesis although not infertility in F2 offspring

(Newbold, et al., 2000). These effects were ascribed by the authors as potentially being due to gene imprinting (Li, et al., 1997, Newbold, et al., 2000) although the formation of DNA adducts leading to mutation in gametic stem cell lines is also discussed. A1221 impairs fertility and embryogenesis in a mouse *in vitro* fertilization model (Kholkute, et al., 1994a), and induces genotoxic effects via intrachromosomal recombination (Aubrecht, et al., 1995) and deletions (Schiestl, et al., 1997) *in situ* and *in vivo*.

We postulate four possible explanations for the transgenerational effects we observed, that are not mutually exclusive: (1) A1221 exposure directly introduced genotoxic effects in the germline of F1 females; (2) A1221 altered epigenetic patterning (e.g., DNA methylation/acetylation) in the F1 embryos, which was then passed to the F2; (3) A1221 caused changes in the behavior of the F1 dams towards their F2 pups, which in turn modified neonatal hormones of the fetuses and subsequently altered steroid hormones; or (4) prenatal A1221 exposure altered circulating hormone levels during pregnancy in the F1, which exposed developing embryos to an improper hormonal environment. These four possibilities are discussed below.

Gross chromosomal aberration (1) caused by PCBs in the developing embryo's germ cell line may have multigenerational effects. A1221 is known to induce mitotic deletion events (Aubrecht, et al., 1995, Schiestl, et al., 1997). One of the major constituents of A1221, PCB5, can increase the incidence of recombination in Drosophila line used to compare carcinogenicity of test substances (Butterworth, et al., 1995). When chromosomal damage occurs in the developing germ line, it has the potential to be transmitted to offspring (Choudhury, et al., 2000). Although A1221 may be causing gametotoxicity in F1 offspring, it is unlikely that this gross chromosomal aberration would result in a uniform F1 or F2 developmental or

physiological phenotype. However, chromosome damage of the germ line may be related to the slight, but non-significant, effects observed on sex ratio in the F2.

Epigenetic alteration of the tertiary structure of DNA (2) involves the addition or removal of methyl or acetyl groups at specific base pair sequences, and causes a long-term alteration in gene expression levels. Epigenetic alterations normally occur at specific stages of somatic and reproductive development, or can be artificially induced by toxin exposure in the adult (Bollati, et al., 2007). Multigenerational transmission of a developmentally induced F1 epigenetic pheotype may be accomplished due to epigenetic patterning of the proto-germ cell line during development. Acute exposure of embryonic male rats to the anti-androgen fungicide vinclozolin (Anway, et al., 2005, Chang, et al., 2006) or the estrogenic substance methoxychlor (Anway, et al., 2005) during the period of male gonadal sex determination causes decreased sperm count and increased occurrence of infertility in the exposed generation. The offspring of these male rats exhibited the same detrimental phenotype for at least three generations, the extent of the experiments. In the case of vinclozolin, this effect was attributed to epigenetic alteration of gene methylation in at least 15 candidate genes tested (Chang, et al., 2006).

In addition to germ cell alteration, poor or abnormal maternal care due to PCB exposure during pregnancy can result in epigenetic changes in somatic DNA metastructure (3) of subsequent generations. For example, rat pups receiving substandard maternal care from a dam undergo epigenetic patterning of glucocorticoid receptor (Weaver, et al., 2004). Simultaneously, estrogen receptors alpha and beta in the preoptic area of the brain are epigenetically imprinted, leading to decreased adult expression of their protein products (Champagne, et al., 2006). Multigenerational transmission

of the epigenetic change has not been verified, however female pups raised by a genetically unrelated dam showing poor maternal behaviors will similarly exhibit poor maternal care for her offspring (Champagne and Meaney, 2001), suggesting the potential for multigenerational transmission of this epigenetic alteration.

Certain toxicants can alter hormone signaling, and when this occurs during pregnancy, a second generation may be affected (4). During the period of PCB administration in this experiment, on E16 and E18, circulating estradiol and prolactin levels are low, and circulating progesterone levels are high (Rosenblatt, et al., 1994). Introduction of an endocrine active substance such as PCBs during this period has the potential to alter endogenous hormone levels. Exposure of pregnant rats to 17α-ethinylestradiol slightly earlier in the gestational period, from E9-E14, can alter maternal behaviors and induce anxiety-like behaviors in offspring (Arabo, et al., 2005). Prenatal exposures to nicotine can induce age-delayed impairment in ovarian and uterine steroidogenesis with implications for next-generation development (Holloway, et al., 2006).

It is likely that several of these mechanisms contribute to the multigenerational effects of A1221 exposure. Epigenetic patterning and altered maternal care of offspring can both be influenced by, or can themselves alter hormone signaling. Thus, while observed effects on altered circulating hormones and gonadal weights in second generation-exposed females are likely due to epigenetic patterning or an improper embryonic hormonal milieu, trends in litter size and sex ratio may be influenced by genotoxicity of oocytes in developmentally exposed F1 females.

Conclusion

We have demonstrated that prenatal exposure to low-levels of Aroclor 1221, a lightly-chlorinated PCB mixture and environmental toxicant, has complex effects on physiology, fertility, and fecundity spanning two generations. The most salient results from this study include altered uterine weights and circulating hormone levels in the second generation consistent with aberrant ovulatory processes that may diminish reproductive success. The finding of multigenerational effects is important because it signals that halting production of a toxic substance such as PCBs is not sufficient to prevent further health risks. Altered sex hormone regulation in the second generation may also negatively impact fertility and fecundity and could induce abnormal development of further generations.

Research Section 2:

The effects of prenatal PCBs on adult female paced mating reproductive behaviors in rats

Abstract

Polychlorinated biphenyls (PCBs) are a family of toxicants that persist in measurable quantities in human and wildlife tissues, despite a ban on their production in 1977. Some PCB mixtures can act as endocrine disrupting chemicals (EDCs) by mimicking or antagonizing the actions of hormones in the brain and periphery. When exposure to hormonally active substances such as PCBs occurs during vulnerable developmental periods, particularly prenatally or in early postnatal life, they can disrupt sex-specific patterning of the brain, inducing permanent changes that can later be manifested as improper sexual behaviors. Here, we investigated the effects of prenatal exposure to the PCB mixture Aroclor (A) 1221 on adult female reproductive behaviors in a dose-response model in the Sprague-Dawley rat. Using a paced mating paradigm that permits the female to set the timing of mating and control contact with the male during copulation, we were able to uncover significant differences in female-typical sexual activities in A1221-exposed females. Specifically, A1221 causes significant effects on mating trial pacing, vocalizations, and the female's likelihood to mate. Furthermore, the intermediate treatment group has the greatest number of disrupted endpoints, suggestive of non-linear dose-responses to A1221. These data demonstrate that the behavioral phenotype in adulthood is disrupted by low, ecologically relevant exposures to PCBs, and the results have implications for reproductive success and health in wildlife and women.

Introduction

Polychlorinated biphenyls (PCBs) were used in industry as inflammable coolants and lubricants and as components of paints and plastics. Banned in 1977 in response to dawning public awareness of their estrogenic and potentially toxic effects on humans and wildlife, PCBs continue to leach into soil, air, and groundwater via retired industrial equipment, and from old factories and buildings. PCBs may have variable degrees of impact depending on which congeners or congener mixtures are involved, the organism's age at exposure, the sex of the individual, the degree of exposure, and the availability of compensatory diet or social buffering to counteract those effects. An accurate evaluation of ecologically relevant xenobiotic exposure depends on the close examination of PCB exposures at low doses (Battershill, 1994, Brouwer, et al., 1999).

The neuroendocrine system serves as an interface between the central nervous system and peripheral endocrine organs, and thus represents a prime target for endocrine disruption by PCBs (Patisaul, et al., 2006). PCBs and their metabolites can act at multiple nodes of the neuroendocrine axis: they may serve as hormone mimics (Connor, et al., 1997), alter circulating hormone levels (Desaulniers, et al., 1999), change patterns of estrous cyclicity (Meerts, et al., 2004a), disrupt hormone metabolism (Gregoraszczuk, et al., 2005, Kester, et al., 2000, Yamane, et al., 1975), influence endocrine-related and hypothalamic gene expression (Aluru, et al., 2004, Bansal, et al., 2005, Colciago, et al., 2005, Flouriot, et al., 1995, Gore, et al., 2002, Pravettoni, et al., 2005, Salama, et al., 2003), interfere with hormone binding proteins (Brouwer and Van Den Berg, 1986, Chauhan, et al., 2000), alter neuronal signaling to endocrine regions of the brain (Khan and Thomas, 2001, Morse, et al., 1996b, Seegal, et al., 1985,

Seegal, et al., 1990) or indirectly affect steroid receptor availability via molecular crosstalk (Brunnberg, et al., 2003, Pearce, et al., 2004).

The behavioral phenotype is perhaps the most sensitive and salient measure of PCB disruption of the neuroendocrine system, because reproductive success hinges upon the normal complement of reproductive behaviors. Previously, PCBs and their metabolites were shown to impact neurotransmitter and steroid hormone systems underlying reproductive function (Khan and Thomas, 2001, Ptak, et al., 2005, Seegal, et al., 1985, Seegal, et al., 2002, Tsai, et al., 1997). These changes in turn are likely to have profound effects on reproductive behaviors. Moreover, the timing of exposure to PCBs is key to the severity of the reproductive phenotype. In particular, exposure during the critical period of brain sexual differentiation is potentially detrimental. This critical period in rats has been proposed to begin in the third trimester of pregnancy and end shortly after birth, from approximately embryonic day 16 to postnatal day (P) 5 in rats (Becu-Villalobos, et al., 1997, Breedlove, 1992, Dohler, 1991, Rhees, et al., 1990, Tobet and Fox, 1989, Wagner, et al., 1998) although a revisitation of brain sensitivity to steroid hormones suggests that the critical period may last longer into postnatal life than previously thought (Primus and Kellogg, 1990, Romeo, 2003). Nevertheless, it is clear that late gestation, the time during which we administered PCBs in our current study, is an important critical window in the organization of sex-typical behaviors (Perakis and Stylianopoulou, 1986). It represents a period during which hypothalamic GnRH neurons are developing (Aubert, et al., 1985), hypothalamic and preoptic area estrogen receptor alpha expression increases (Pasterkamp, et al., 1996), and sexually dimorphic progesterone receptor expression is determined (Chung, et al., 2001, Wang, et al., 2002).

In the present study, we investigated the effects of prenatal exposure to the PCB mixture Aroclor (A) 1221 on adult female reproductive behaviors, using a dose-response model encompassing ecologically-relevant exposures, and a paced mating paradigm to uncover feminine reproductive behaviors in the Sprague-Dawley rat. Our results show specific impairments in several feminine sexual behaviors that have implications for reproductive success.

Materials and Methods

ANIMALS

All experimental procedures were performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Timed pregnant Sprague-Dawley rats were purchased from the University of Texas Animal Resources Center, and were housed individually under a 12:12 light cycle. Animals were fed low-phytoestrogen rat chow (Harlan Teklad Global Diet 2019) and water *ad libitum*. Pregnant dams were intraperitoneally injected with 0.1 ml of vehicle (dimethyl sulfoxide, DMSO) or Aroclor 1221 (Accustandard #C-221N-50MG; Lot#072-202, reconstituted in DMSO) at one of three doses (0.1, 1, or 10 mg/kg), on E16 and E18, the third trimester of pregnancy in rats. Intraperitoneal injection was chosen as a mode of administration to eliminate possible variability in gastrointestinal absorption via oral exposures, as per the experimental methods of other published studies of PCB effects (Chung and Clemens, 1999, Gillette, et al., 1987, Murugesan, et al., 2005).

PCBs administered to the dam are not fully transferred to offspring, either during gestation or via lactation, and amounts transferred to each pup are estimated to be approximately 500x less than maternal exposure (Takagi, et al., 1986). Therefore, we estimate that our pups were exposed to

a maximum of 0.2, 2 and 20 μg/kg, in the range of estimated human exposures (Lackmann, 2002). Impregnated dams were handled throughout the first few weeks of gestation to minimize stress of handling during injections, and a two-person method of injection was used to ensure accurate localization of the injection and to decrease stress. For this method, one person gently held the rat and a second person administered the drug; rats remained calm through the procedure due to extensive handling experience. Nesting materials were provided on gestational day 20, and the day of birth was recorded as P0. Pups were not handled until P1 in order to reduce stress to the dams and pups, and to avoid the possibility of interrupting parturition.

On P1, litters were culled to 4 female pups per litter (or fewer if litters had fewer females) in order to minimize inter-litter variability. Whereas culling to single-sex litters may impact the development of normal sociosexual behaviors (Moore and Morelli, 1979, Pellis and Pellis, 1997, Sharpe, 1975), dams show suckling and grooming preferences that differ depending upon sex ratio (Crews, et al., 2006, Szyf, et al., 2005). Rats were weaned on P22 to 3-4 littermates/cage. Daily vaginal smears were conducted to determine estrous cyclicity following puberty. The control (vehicle), 0.1 mg/kg, 1 mg/kg, and 10 mg/kg A1221 treatment groups contained, respectively, 11, 11, 10, and 10 litters, and 3-4 females were used per litter for behavioral tests described below.

PACED MATING CAGE

Female sexual behaviors were tested using a paced mating protocol (Coopersmith and Erskine, 1994, Erskine, 1985, Paredes and Vazquez, 1999). Paced mating cages were constructed of a 30” long x12” wide x 17” tall Plexiglas aquarium fitted with a clear Plexiglas panel bisecting the cage

lengthwise. Two 1.75” diameter openings at the base of this panel allow the female to pass between the two chambers, however, the males were too large to fit through the openings. The side of the cage that restricts the male is hereby referred to as the “mating chamber” and the other side is called the “escape chamber”. Figure 9 shows an image of the cage.

Figure 9: Paced Mating Cage

Figure 9. The paced mating cage is a Plexiglas aquarium bifurcated by a clear Plexiglas divider containing two holes equally spaced from the midline. The female, on the right in the escape chamber, is small enough to easily pass through either opening. The male, shown at the far corner on the left in the mating chamber, is too large to fit and thus remains restricted to the mating chamber.

CRITERIA, PREPARATION FOR, AND ANALYSIS OF TRIALS

Paced mating experiments commenced when the females reached P50. Experimental trials were conducted starting at 5 hours after lights out under dim red lighting, on the evening of proestrus, a time when females are sexually receptive (Barfield and Lisk, 1970). Sexually experienced but otherwise experimentally untreated males (7-11 months) were used. Rats were habituated to the mating cage at least twice prior to the experiment for 20 minutes (males) or 10 minutes each (females). One hour prior to each experiment, activity levels (ambulation) of experimental females were tested by allowing the female to roam freely throughout the mating cage for 10 minutes. The baseline ambulation rate determined by counting the number of times the female crossed the panel during the 10 minute period. This pretrial ambulation value was later used to normalize the mating trial ambulation. Males were placed into the mating chamber for at least 20 minutes directly preceding each mating trial, after which a solid opaque divider was placed between the chambers. The experimental female was then introduced to the escape chamber, and the opaque divider was lifted to begin the trial. Paced mating parameters were scored for statistical purposes through seven intromissions (Edmonds, et al., 1972, Erskine, et al., 2004). This choice was made because female rats receiving between 5 and 10 intromissions are most sensitive to mating trial pacing in the establishment of pregnancy (Erskine et al., 2004). As the number of intromissions to ejaculation was variable we chose seven as a standard number for most analyses, and it falls within this range of 5 to 10. In addition, trials were allowed to proceed to the first ejaculation so that post-ejaculatory behaviors could be recorded, but non-ejaculatory events following the seventh intromission were not included in analysis, or in calculating trial length. After each trial, the male and female

were placed in a housing cage and allowed to continue mating overnight to reinforce the behavior for the males.

Females that did not show lordosis within 20 minutes in the presence of a sexually active male were permitted 3 additional mating trial opportunities that always occurred on the evening of proestrus. After four unsuccessful mating trials, females were eliminated from the experiment and were excluded from analyses. In order to eliminate any effect of the male, the same male animals were mated at different times with females from control and all three doses of PCB-treated groups. Males were given 2-3 day intervals between mating trials to prevent fatigue. The day following a successful mating trial, vaginal smears were conducted to determine the presence of sperm, and vaginal cytology was recorded. Females were then weighed and euthanized via decapitation.

Trials were recorded on videotape for later review by the experimenter, who was blind to the rats' identities. Following completion of mating trial event logs, treatments were decoded and analysis was performed on the following parameters (described in Table 7): Male-typical behaviors, mating trial pacing, avoidance/rejection behaviors, ambulation, and receptivity. When calculating total time for the mating trial, post-ejaculatory refractory period was not included. Female vocalizations were recorded as the number of audible vocalizations per minute. When a female failed to vocalize during a mating trial, she was given a score of 0, which was factored in for statistical analysis.

Table 7. Paced Mating Behaviors

Mating Trial Pacing	
Mount-return latency	Mean time between a mount and the female's return to the mating chamber
Intromission-return latency	Mean time between an intromission and the female's return to the mating chamber
Ejaculation-return latency	Mean time between an ejaculation and the female's return to the mating chamber

Avoidance and Rejection Behaviors	
% Time in escape chamber	Time in the escape chamber/trial length
Rejection quotient	(# lateral kicks + # face-to-face's)/# mounts
Vocalizations/time	# audible vocalizations/trial length

Ambulation	
Pretrial activity level	# crossings of empty mating cage/10 minute period
Trial activity level	# crossings of mating cage/trial length
Normalized activity levels	Trial activity level/pretrial activity level

Receptivity and Proximity Behaviors	
Lordosis quotient (LQ)	# lordosis/# mounts
Mount % leave	# times female leaves mating chamber after a mount/ # mounts
Intromission % leave	# times female leaves mating chamber after an intromission/# intromissions
# Attempts	# mating trial attempts

Male-Typical Behaviors	
Mount frequency	# mounts/trial length
Intromission frequency	# intromissions/trial length

STATISTICS

The number of animals tested in the control, 0.1, 1, and 10 mg/kg groups were 35, 40, 32, and 35, respectively. We first determined whether each endpoint was normally or non-parametrically distributed, and then carried out the following analysis: with the statistical analysis software SAS (Littel et al., 1999), we completed a simple linear mixed model ANOVA analysis including a fixed treatment effect and a random dam (treatment) effect. In addition, we included a fixed covariate of the log transform of mating trial length to control for mating time. These models were estimated using restricted maximum likelihood and statistical significance was determined by a z-score or an F-value for the random and fixed terms, respectively. A number of the phenotypes were non-normally distributed and so were transformed using a log (1+y) transformation. For very highly skewed traits, we employed a non-parametric permutation testing procedure (Cassell, 2002). In this case, the phenotypes were randomized with respect to the experimental effects 1000 times and the analyses were completed as above. Here, the distribution of the test statistics under the null hypothesis was empirically determined with alpha set at 0.05. If significant treatment effects were observed, we used a series of *post-hoc* tests for all combinations of treatments. Here we controlled for multiple tests with a Tukey-Kramer adjustment. For data presentation, raw data are shown, and statistically significant differences calculated as above are indicated.

Results

MATING TRIAL PACING

Results on latencies for females to return to the mating chamber after mounts, intromissions, and ejaculations are shown in Figure 10. Of the behaviors scored, mount-return latency ($F(3, 38) = 3.53$, $p < 0.05$) and the post-ejaculatory interval ($F(3, 38) = 3.23$, $p < 0.05$) were significantly affected by A1221 treatment ($p < 0.05$ for both). *Post-hoc* analyses of mount-return latency revealed that the 1 mg/kg group had a longer mount-return latency than the 0.1 mg/kg group ($p < 0.05$). For post-ejaculatory return interval, *post-hoc* analysis showed that the 1 mg/kg group had a longer post-ejaculatory interval than the 10 mg/kg treatment group. No groups were significantly different from control.

Figure 10.

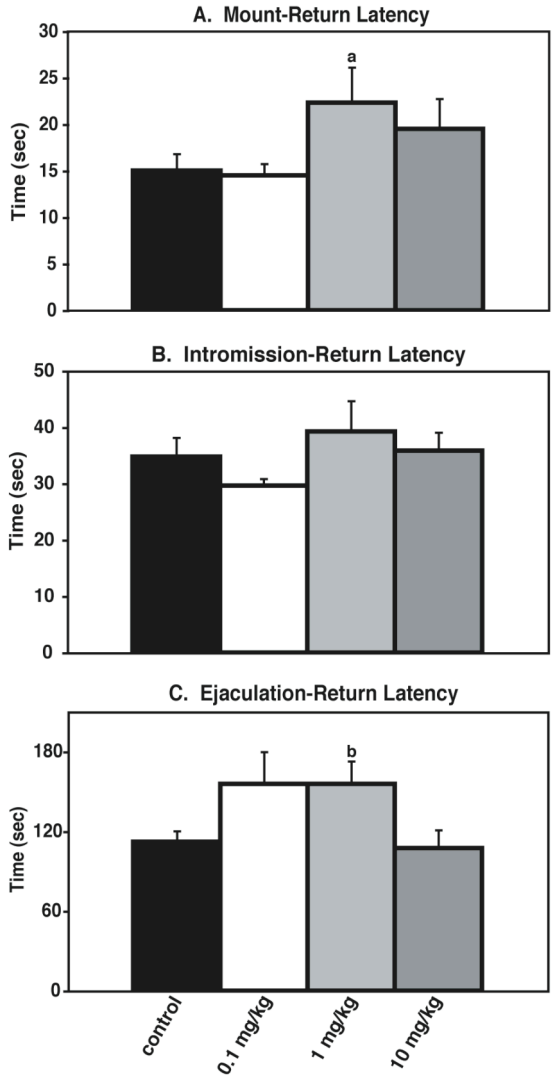


Figure 10. Mating Trial Pacing. A. Mount Return Latency: A significant effect of treatment was found ($p < 0.05$), with significant differences between the 1 mg/kg and the 0.1 mg/kg group ($p < 0.05$), designated by (a). B. Intromission Return Latency: No significant differences were detected. C. Post-ejaculatory Return Latency: A1221 significant altered this parameter ($p < 0.03$), with *post-hoc* analysis confirming significance between the 1 mg/kg and 10 mg/kg group ($p < 0.03$), designated by (b). Data shown in this and subsequent figures are mean ± SEM.

RECEPTIVITY AND PROXIMITY BEHAVIORS

Neither lordosis quotient (Figure 11A), nor the percentage of mounts and intromissions followed by the female leaving the mating chamber (percent exits after mount and percent exits after Intromission, respectively) differed between treatment groups (Figure 11B & 11C). By contrast, when effects of A1221 on the number of trials required for a proestrous female to exhibit receptivity was quantified, a highly significant effect was found ($F(3, 38) = 4.84$, $p < 0.005$; Figure 11D). *Post-hoc* analyses showed that rats in the 1 mg/kg A1221 dose required significantly more trials to mate successfully compared to control rats ($p < 0.005$). In addition, rats in both the 1 mg/kg and 10 mg/kg groups required significantly more mating trials than the 0.1 mg/kg group ($p < 0.01$; $p < 0.05$, respectively).

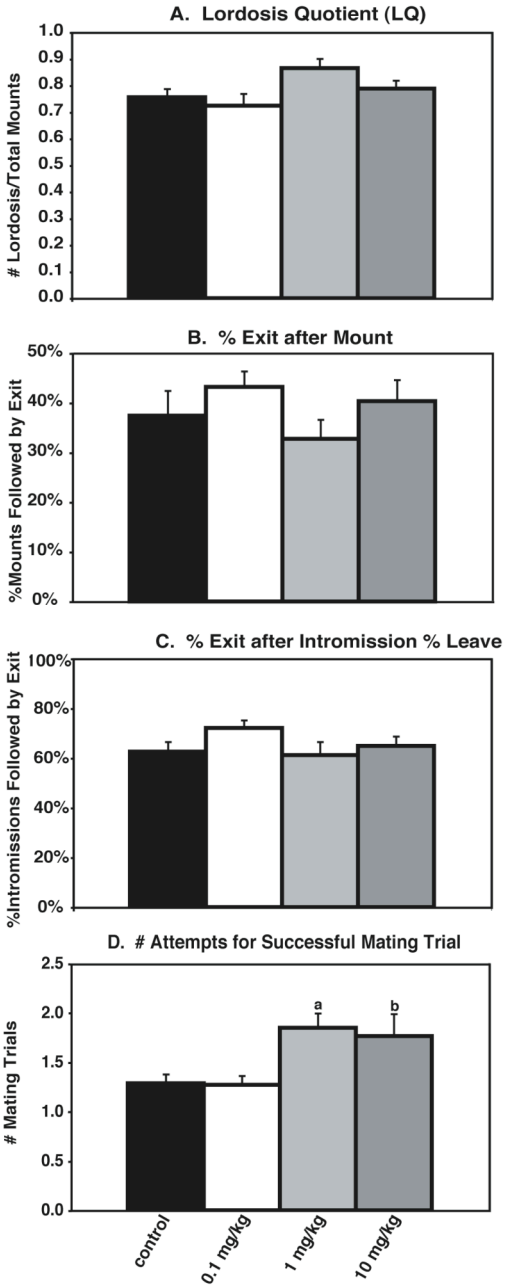


Figure 11. Receptivity. A. Lordosis Quotient: No significant effects were found. B. Percent exits after mount: No differences among groups were detected. C. Percent exits after intromission: There were no significant differences among groups. D. Number of Attempts Required for a Successful Mating Trial: There was a significant difference between groups at $p < 0.01$. *Post-hoc* analysis showed that the 1 mg/kg group required significantly more mating trials before being receptive to a male compared to control ($p < 0.005$) and 0.1 mg/kg treated rats ($p < 0.01$). These differences are designated by (a). The 10 mg/kg group also required more mating trials than the 0.1 mg/kg group ($p < 0.05$, designated by (b)).

AVOIDANCE/REJECTION BEHAVIORS

Three behaviors were evaluated as avoidance/rejection behaviors: the percent time the female spent away from the male in the escape chamber, the numbers of kicks and face-to-face behaviors, and the number of vocalizations in the audible range, the latter a potential index of stress (Han, et al., 2005). There were no significant differences between groups for the percentage of time in escape chamber (Figure 12A) or for rejection quotient (Figure 12B). A statistically significant effect was found for female audible vocalizations ($F(3, 38) = 2.53$, $p < 0.05$; Figure 12C), and *post-hoc* analysis found that the 1 mg/kg group vocalized significantly less than either the control or the 0.1 mg/kg groups ($p < 0.05$ for both comparisons).

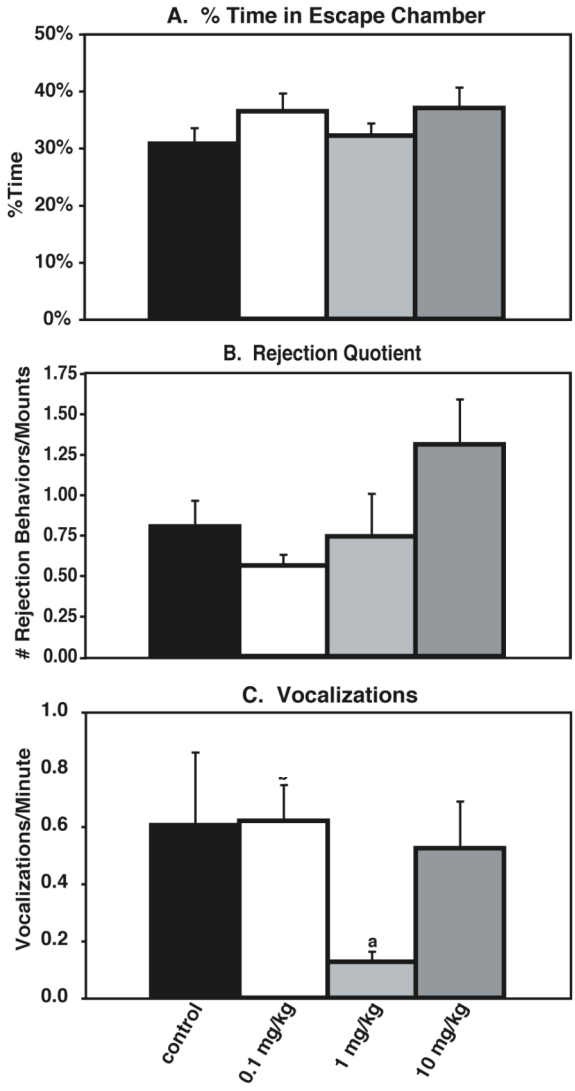


Figure 12. Avoidance/Rejection Behaviors. A. Percent Time in Escape Chamber: All of the treatment groups spent approximately the same percentage of the mating trial away from the male in the escape chamber. B. Rejection Quotient: The number of face-to-face and lateral kicks in a mating trial divided by total mounts, did not differ among groups. C. Vocalizations: Statistical analyses showed an overall main effect of treatment ($p < 0.05$). *Post-hoc* analysis demonstrated that the 1 mg/kg had significantly fewer vocalizations than control ($p < 0.05$,) and 0.1 mg/kg ($p < 0.05$); these differences are designated by (a).

AMBULATION

Although pretrial ambulation levels did not differ significantly among groups (Figure 13A), when mating trial ambulation was normalized to pretrial levels, a significant difference was detected ($F(3, 38) = 3.01$, $p < 0.05$). However, *post-hoc* analyses found no significant interactions, although the 10 mg/kg group showed a non-significant trend for reduced activity compared to the 0.1 mg/kg group ($p < 0.07$; Figure 13B).

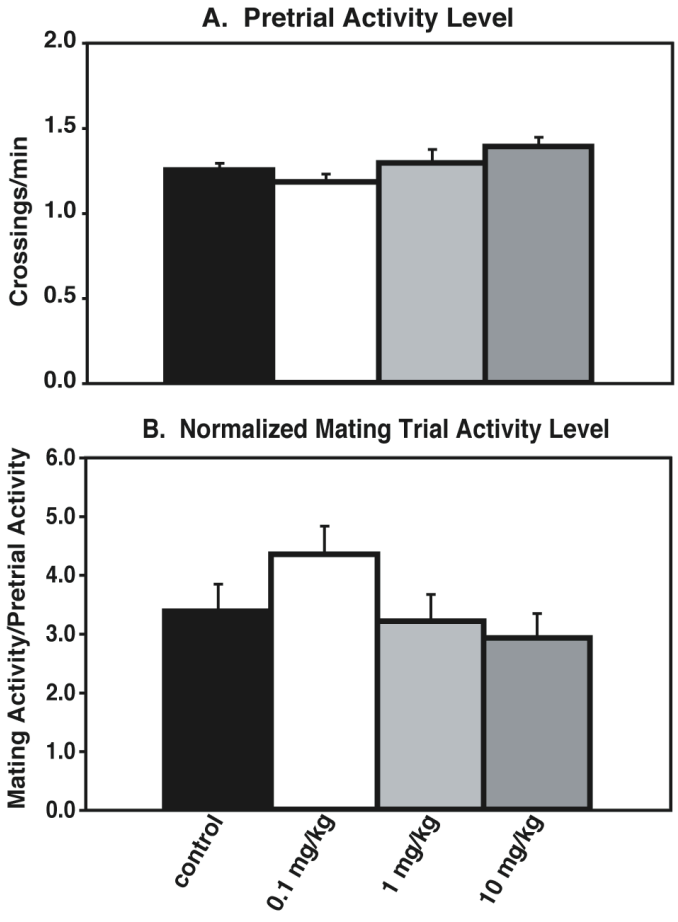


Figure 13. Ambulation. A. Pretrial ambulation: Female activity levels were measured at least one hour prior to mating trial commencement in a paced mating cage in the absence of the male. No differences were detected. B. Normalized Mating Trial Ambulation Level: Mating trial activity was normalized to pretrial values, and a significant main effect was found ($p < 0.05$). However, *post-hoc* analysis found no significant differences between groups.

MALE-TYPICAL BEHAVIORS

The same males were rotated through mating trials across all groups of females to minimize any effects of inter-male variability. We observed no statistically significant differences between any combination of treatment groups for mount frequency or intromission frequency of males towards females among the different treatment groups (Table 8).

Table 8. Male-typical behaviors.

	Control	**0.1 mg/kg**	**1 mg/kg**	**10 mg/kg**
Mount Frequency	1.40 ± 0.08	1.39 ± 0.13	1.41 ± 0.16	1.28 ± 0.13
Intromission Frequency	0.68 ± 0.05	0.75 ± 0.05	0.80 ± 0.09	0.71 ± 0.06

Mount frequency and Intromission frequency are shown as number of events per minute. Neither parameter varied significantly among treatment groups. Data shown are mean $\pm$ SEM.

Discussion

We investigated the effects of prenatal PCB exposures on adult female sexual behaviors and observed several endpoints that were significantly altered by A1221 exposure. Overall, we observed the greatest number of effects of the intermediate (1 mg/kg) dosage of A1221, exposure to which affected adult mating trial pacing, receptivity/proximity behaviors, and audible vocalizations compared to control and lower or higher doses of A1221. This suggests the possibility of a non-linear dose response curve common to endocrine disruption studies (Calabrese and Baldwin, 2001a), wherein an intermediate dose level elicits the greatest effects. Thus, the paced mating paradigm reveals that small, ecologically relevant exposures at a precise developmental stage can permanently alter specific aspects of adult female-typical sexual behaviors.

In 1999 and 2001, two papers were published reporting that perinatal (embryonic day (E) 14, P0 and P10; Chung and Clemens, 1999), but not postnatal (P1-7; Chung and Clemens, 2001) exposure of rat dams to the commercial PCB mixture A1221 alters sexual behaviors in the female offspring in adulthood. Our current study follows up upon and extends the work of Chung and Clemens in several novel ways. First, we focus on effects of prenatal A1221 administered to the dams during the third trimester of gestation to clarify the role of this developmental window on latent reproductive behaviors. Second, we use gonadally-intact as opposed to ovariectomized females. Third, we use a slightly lower and broader range of exposures (0.1, 1 and 10 mg/kg) to approximate human and wildlife exposure levels and to investigate the possibility of a non-linear dose response curve (Gore, et al., 2006b, Weltje, et al., 2005). By comparison, the dosages used by Clemens' laboratory ranged from approximately 8 to 42 mg/kg. Fourth, and most importantly, we investigated some endpoints that, to

our knowledge, have not been investigated by other laboratories. Our most robust novel findings were of significant effects of A1221 on audible vocalizations, a potential index of stress during mating, and on the number of trials required to mate successfully. These latter results and their implications are discussed in more detail below.

PROPERTIES OF A1221 AS AN ENDOCRINE-DISRUPTING CHEMICAL

A1221 has previously been shown to exert actions on endocrine systems. It alters numbers of estrogen receptor beta immunoreactive cell numbers in the anteroventral periventricular nucleus of the female rat brain, a region important for ovulation in rats (Salama, et al., 2003). In addition, A1221 interferes with other endocrine systems and functions including, but not limited to: the thyroid neuroendocrine axis (Kilic, et al., 2005); aromatase activity *in vitro* (Woodhouse and Cooke, 2004); it acts as an androgen receptor antagonist *in vitro* (Schrader and Cooke, 2003); it is estrogenic in estrogen-sensitive cell preparations (Shekhar, et al., 1997); and it can inhibit fertilization of the mouse oocyte (Kholkute, et al., 1994b). Together, these data suggest the potential for a wide spectrum of A1221 actions on endocrine systems.

A1221 is a very lightly chlorinated PCB mixture containing primarily mono- and ortho-substituted congeners. A1221 is more volatile than more heavily chlorinated PCB mixtures, and exerts more transient effects (Thomas, et al., 1998b), making it difficult to reliably measure in biological samples (Frame, 1997b). Although to our knowledge, tissue burden analysis has not been conducted specifically for the commercial mixture A1221, half-life analysis has been conducted on other lightly chlorinated PCBs, supporting a positive correlation between degree of chlorination and half-life (Matthews and Anderson, 1975), with the shortest half lives, associated with

the lowest chlorinated congeners, being in the range of several days (Tanabe, et al., 1981). Our experimental animals were treated *in utero* with low doses of PCBs approximately 5 days prior to birth, and were euthanized at approximately 60 days, making it highly unlikely that detectable PCBs persist. Nevertheless, our results demonstrate a long-lasting effect following developmental exposures. This is most likely due to interference in normal developmental patterning during the late embryonic sensitive period for neuroendocrine sexual differentiation.

As reported previously for A1221 and other PCBs, effects on endocrine systems are often non-linear (reviewed in Gore et al., 2006), and our results support such findings. For the two endpoints that differed between A1221 and control rats, namely, the number of opportunities required for successful mating, and audible vocalizations, we observed the greatest effect on the intermediate dose of A1221, 1 mg/kg, compared to the control group. In addition, we observed several other significant differences between treatment groups. These latter observations demonstrate how employing a range of dosages is informative in revealing complex effects of environmental endocrine-disrupting chemicals.

A1221 EFFECTS ON PACED MATING BEHAVIORS

The paced mating paradigm enables the female rather than the male to control the pattern of mating. Not only is this paradigm considered most "rewarding" for females as measured by place preference for the location where paced mating occurred (Paredes and Vazquez, 1999), but it also increases fecundity as evidenced by the average number of pups/litter (Coopersmith and Erskine, 1994). Here, we observed several significant effects of prenatal exposure to A1221 on female mating trial pacing that varied by dosage, with the intermediate 1 mg/kg dosage of A1221 exerting

the strongest effects. One of our most robust findings was that females exposed prenatally to A1221 at 1 mg/kg required a greater number of opportunities before they would mate than either the control or the 0.1 mg/kg groups. The 10 mg/kg treatment group was also affected, requiring more mating trials than the 0.1 mg/kg group to mate successfully.

Proximity (event-return) behaviors, which measure the female's choice to remain with a male after a mating event and are revealed in the paced mating model, were significantly affected in A1221 rats, specifically for mount-return and post-ejaculatory-return latencies, which were longest in the 1 mg/kg group. Event-return latencies are believed to be a function of the intensity of vaginal stimulation by the male's pelvis and penis (Erskine, 1992, Wersinger, et al., 1993), and PCBs can potentially alter vaginocervical sensation by affecting peripheral nerve conduction, as has been shown in humans (Chen, et al., 1985). A longer event-return latency may thus be an indicator of altered vaginal sensitivity in the intermediate A1221 group. Our results suggest that A1221 may alter the amount of time that females choose to spend with males after a mating event. Consistent with this, other reports on PCBs have shown effects on other sensory and motor parameters in many systems, including audition (Crofton, et al., 2000) sensory and motor nerve conduction velocities (Chen, et al., 1985), and vision (Kremer, et al., 1999).

Not all aspects of the suite of mating behaviors were significantly affected by early PCB treatment. We did not detect significant differences in lordosis quotient (LQ) among treatment groups, although there was a non-significant trend for the highest LQ in the 1 mg/kg group. Similarly, percent exits after mounts and intromissions were unaffected. These results indicate that only specific behaviors, and presumably specific neural circuits underlying these behaviors, are altered by fetal A1221 exposure.

The sexual behaviors of females used in this study were tested beginning at age P50. Female rats achieve puberty at approximately 32-38 days of age (See Research Section 1 of this dissertation) after which they are sexually mature. In the wild, animals are probably impregnated very early in adulthood (Mcclintock, 1983). Mating trial pacing by the female rat is directly related to successful impregnation (Erskine, et al., 2004), and sexual behaviors of aged females are comparable to those of young female rats (Cooper and Linnoila, 1977). To my knowledge, the effect of sexual experience on female paced mating parameters has not been investigated. Our findings that A1221 significantly alter specific aspects of the timing of paced mating behaviors has relevance to wildlife exposed to endocrine-disrupting chemicals, as such animals may not get a second chance to mate and reproduce in order to ensure transmission of their genes to subsequent generations.

EFFECTS OF A1221 ON AUDIBLE VOCALIZATIONS

Audible rat vocalizations, in the audible human frequency range and with a fundamental frequency of approximately 2.5 kHz, are indicative of stress or pain, especially at increased temporal frequencies (Han, et al., 2005). Audible rat vocalizations, in the human frequency range, are indicative of stress or pain, especially at increased temporal frequencies (Han, et al., 2005). Audible vocalizations, but not ultrasonic vocalizations were recorded in the methodology of this experiment. Rat audible vocalizations are commonly studied as a response to Pavlovian conditioned stress, or pain anticipation (Borszcz, 1995). Rat ultrasound vocalizations at 22 KHz are similarly associated with stressful or noxious stimulation (Calvino, et al., 1996, Kaltwasser, 1990, Vivian and Miczek, 1993), however we did not record this type of vocalization for the current study. The

production of the 22 Khz call is specific to only a subset of noxious/stressful situations (Jourdan, et al., 1995, Wallace, et al., 2005) and there is no temporal correlation between vocalization at this frequency and stimulus presentation (Jourdan, et al., 1995). In one study directly comparing audible vocalizations with 22 KHz calls, analgesic administration during the application of nociceptive tail shocks reduced the intensity of only audible vocalizations, whereas ultrasonic 22Khz vocalizations were not affected (Jourdan, et al., 1998). This body of research suggests that audible vocalizations are an appropriate and sufficient vocal measure of pain or stress in experimental situations.

An unexpected outcome of our study was that the 1 mg/kg PCB group vocalized significantly less than the control and 0.1 mg/kg groups, suggestive of a decreased stress response. Although to our knowledge no studies have reported on another ethological relevance for rat audible vocalizations aside from the pain/stress response, we cannot eliminate the possibility that audible vocalizations during mating serve a previously undocumented social function. Furthermore, the female rats employed in this experiment, although sexually mature, were younger than those typically employed in studies of female sexual behaviors, and their vocalization pattern may differ from the adult; whether or not this is the case, it does not undermine the relevance of PCB exposural effects on this endpoint. PCBs are associated with decreased glucocorticoid levels in rat (Durham and Brouwer, 1990), a human *in situ* system (Li and Wang, 2005), an avian model (American Kestrel) (Love, et al., 2003), polar bears (Oskam, et al., 2004) and rainbow trout (Aluru and Vijayan, 2006). Our results suggest that A1221 may be decreasing stress responsiveness in the 1 mg/kg exposed females. Interestingly, this is the same group that required more trials to successfully mate. The two apparently divergent effects of A1221 on the decreased

likelihood to mate and decreased stress response of the female are likely not part of a cause-effect relationship, but rather, due to differential effects of PCBs on the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal neuroendocrine axes.

A1221 EFFECTS ON OTHER BEHAVIORS

We tested ambulation in the mating cage, both prior to mating and during mating trials. This variable relates possible neurodevelopmental effects of prenatal PCB exposure on hyper/hypoactivity. Although treatment did not affect activity prior to the mating trial, activity during the trial itself was significantly different between groups; *post hoc* analyses did not reveal specific differences between groups, although there was a trend for increased activity in the 0.1 mg/kg group. The overall effect of treatment on activity is consistent with previous reports on PCB exposure in rhesus monkey and rats (Bowman, et al., 1981, Holene, et al., 1995, Kuriyama and Chahoud, 2004, Lilienthal, et al., 1990). Effects of A1221 on time spent in the escape chamber did not differ among groups, nor was there any effect on rejection quotient. Thus, again, effects of A1221 appear to be specific to subsets of behaviors.

Conclusion

Studies in humans and wildlife reveal that environmental exposures to polychlorinated biphenyls continue to be ubiquitous across a broad geographical range. This study on Sprague-Dawley female rats demonstrates that exposure to low levels of A1221 during a prenatal developmental window of brain sexual differentiation is sufficient to induce latent changes in a subset of adult reproductive behaviors. Specifically, at PCB exposures approximating those of wildlife and humans, the number of

trials required for successful mating was compromised. In addition, abnormal audible vocalization behavior during mating may suggest that the stress system may be affected in PCB-exposed females. These results have potential implications for reproductive disorders of women and endangered animals.

Research Section 3:

The effects of prenatal PCBs on adult female gene expression in the preoptic area of the brain

Abstract

The production and commercial use of novel chemicals over the past century has—introduced toxicants into the environment for which few natural degradatory mechanisms exist. One class of environmental toxicants, polychlorinated biphenyls (PCBs), can alter a multitude of physiological processes when encountered by humans and wildlife. The specific biotoxicological effects of PCBs vary depending upon developmental or adult exposure, and with the amount and duration of chemical exposure. The present study investigated the effects of low, ecologically-relevant exposure of the PCB mixture Aroclor (A)1221, given prenatally, on adult gene expression in the preoptic area (POA) of the brain, a region involved in reproductive neuroendocrine physiology and behavior. By using the whole genome Affymetrix 230 2.0 rat GeneChip Array, we were able to test our hypothesis of a primarily estrogenic effect of A1221 on gene expression, as well as to investigate other possible avenues of disruption. Our results reveal that low levels of A1221 during development primarily alter intracellular and intercellular signaling pathways in the brain, and that this effect in the adult may not be related to A1221's known steroid-hormone like actions.

Introduction

Polychlorinated biphenyls (PCBs) are a species of endocrine disrupting chemical (EDC) constituted of linked biphenyl-ring molecules with chloride substitutions at any combination of 10 available carbons. An industrial product used primarily in capacitors and transformers, PCB production was halted in 1978 in response to growing public recognition of their dangerous impact on human health. However, PCBs continue to enter the environment due to improper disposal and the gradual leaching of landfills. After infiltrating the body, PCBs enter storage reservoirs in blood serum, brain, adipose tissue, and organs, from whence they disrupt a multitude of physiological processes. The developing brain is particularly sensitive to PCB disruption during gestation, when the embryo's blood-brain barrier is weak and open to invasive substances, especially in heavily vascularized regions such as the hormone-concentrated region of the median eminence which lies under the hypothalamus and preoptic area (Johansson, et al., 2006, Peruzzo, et al., 2000). In addition, the nervous and endocrine systems undergo many of their greatest changes during embryonic and early postnatal development, and disruption of these systems by PCBs have been shown to cause permanent dysfunction in adulthood (Brevini, et al., 2005, Salama, et al., 2003, Seegal, et al., 2005).

In an effort to better understand the many systems affected by PCB exposure and resulting interactions between disrupted signaling pathways, the toxicology research community has endeavored to document the varied endpoints of PCB exposure using multiple dose levels and ages of exposure. Recent discoveries have contributed to this goal by highlighting a novel role of PCBs in altering gene expression. PCBs can activate expression of immediate early gene c-jun, (Shimokawa, et al., 2006) and intracellular signaling cascades mediated by c-src (Eum, et al., 2006), as well as alter

DNA binding of transcription factors Sp1 AP1 and NF kappa B (Basha, et al., 2006), all of which can mediate downstream transcriptional events. Altered gene expression following toxicant exposure may predispose an individual to adult disease. For example, prenatal exposure to hydroxylated PCB metabolites can increase adult expression of genes implicated in mammary carcinogenesis (Wakui, et al., 2006). Additionally, adult brain mRNA of genes involved in Parkinson's Disease, including parkin and alpha synuclein, exhibited altered expression following PCB exposure, particularly in the hypothalamus (Malkiewicz, et al., 2006).

Aroclor (A) 1221 is a lightly-chlorinated commercial PCB mixture composed mostly of mono-ortho-substituted and co-planar congeners (Frame, 1997a). The chemical fingerprint of A1221 and its metabolites has been characterized as estrogenic (Layton, et al., 2002, Petit, et al., 1997, Ptak, et al., 2005, Shekhar, et al., 1997), and A1221 or its constituent congeners can alter the ability of aromatase enzyme to convert testosterone to estradiol (Ptak, et al., 2006b, Woodhouse and Cooke, 2004). In addition to perturbation of the steroid hormones, A1221 exposure can mimic thyrotoxicosis and alter circulating thyroid hormone levels (Kilic, et al., 2005). In previous work from our laboratory, A1221 was shown to increase the number of cells expressing estrogen receptor beta protein in the preoptic area of the rat brain after prenatal exposure (Salama, et al., 2003), and to increase GnRH gene expression in immortalized GnRH cell cultures (Gore, et al., 2002).

Here, we investigate the whole genome effects of prenatal A1221 exposure on adult female gene expression in the preoptic area, a neuroendocrine control center adjacent to the hypothalamus. Whole genome microarray analysis is valuable because the immense amount of data produced can be approached in many different ways in order to extract

information that contributes to the formation of new hypotheses. For example, like other environmental toxicants with known endocrine disrupting effects, PCBs often evince a non-linear dose-response curve, an effect known as hormesis (Calabrese and Baldwin, 2001b). By examining gene expression across multiple treatment groups, we can infer whether A1221 is affecting transcription via a hormetic, steroid hormone-like pathway, or via another mechanism, such as disruption of second messenger signaling or direct interference with transcription factor binding at response elements. Functional group analysis and promoter analysis results further characterizeed the genes most affected by exposure. Because of A1221's known endocrine disrupting effects, and the potential for perturbation of downstream transcriptional signaling, we hypothesized that *in utero* exposure during the sensitive period of brain sexual differentiation causes long-lasting alterations of gene transcription into adulthood.

Materials and Methods

ANIMAL CARE

Sprague-Dawley rats were kept on a partially reversed 12:12 hour light cycle (lights on at 23:00 hours) and were fed Harlan-Teklad low phytoestrogen diet (#2019) and given water *ad libitum*. Timed pregnant nulliparous females were given an intraperitoneal injection of 0.1, 1, or 10 mg/kg A1221 or vehicle (98% DMSO) in a volume of 0.1 ml 2 hours prior to lights out on gestational days 16 and 18. The day of parturition, postnatal day (P) 0, was defined as the birth of at least one pup prior to lights-out. On P1, litters were culled to 4 females. Following vaginal opening, female estrous cycles were monitored daily by recording vaginal cytology. Beginning on day P50, females in behavioral estrus, determined by vaginal smears, were allowed to mate with males according to the protocol of a related experiment (Steinberg,

et al., 2007). Experimental animals were euthanized by decapitation approximately 20 hours following a successful mating, 1-2 hours prior to lights-out. The brain was carefully removed from the skull and quickly chilled in crushed ice for 1-2 minutes, after which the preoptic area (POA) was dissected out using an adult rat brain matrix with 1 mm divisions (Ted Pella, model #15007), following anatomical landmarks. Specifically, the brain was placed ventral side up in the matrix, and the insertion place of the optic nerve into the lateral borders of the tuber cinereum was used as the rostro-caudal location of the first coronal cut. Using the brain matrix, the second blade was placed 4 mm rostral to this landmark, and the entire coronal section was removed and placed rostral side up on a clean, chilled glass plate. Then, two parallel cuts were made from just under the midline of the corpus calosum, passing through each lateral branch of the anterior commissure, resulting in an isosceles triangle-shaped section containing the preoptic area. A rat brain atlas was used to confirm accurate dissection (The Rat Brain in Stereotaxic Coordinates, 2nd Edition. George Paxinos and Charles Watson. Academic Press, 1986). The preoptic area was then flash frozen on dry ice prior to storage at -80^{o} C until RNA extraction.

RNA EXTRACTION FOR MICROARRAY

RNA was extracted using a long-established in-house protocol (Daftary and Gore, 2003, Gore and Roberts, 1994, Gore, et al., 1999). Briefly, frozen POA tissues were homogenized via extrusion through a 22 gauge needle, proteinase K was introduced, and RNA was extracted in phenol chloriform and precipitated in ethanol. Nuclear and cytoplasmic RNA were separated using a two buffer system. Extracted RNA was frozen at -80 degrees prior to amplification. The cytoplasmic RNA of three animals per treatment, each

representing a different litter, was chosen for microarray analysis (n = 5, 4, 5, 4 rats in the control, 0.1, 1, and 10 mg/kg treatment groups, respectively).

RNA AMPLIFICATION & HYBRIDIZATION FOR MICROARRAY

Extracted RNA was run on an Agilent Bioanalyzer 2100 to confirm integrity and purity of samples. It was then amplified by Ambion, Inc. (Austin, TX), using the MessageAmp II Biotin kit (Ambion #1791). Amplification products were assessed using the Agilent Bioanalyzer 2100. Product sizes were between 200 and 5000 bases with an average length of 1200 bases. Hybridization of samples to the rat 230 2.0 BioChip, and image capture, were carried out according to Affymetrix protocols. The Affymetrix Statistical Algorithm software GCOSv1.3 was used to quantify image signal results. Hybridization quality control measures including scaling factor (a measure of effective labeling), background noise (relates sample purity), and % Present (referring to the % probe sets detectable, related to sample type and tissue source) were used to confirm accurate hybridization prior to analysis.

ANALYSIS & STATISTICS

Transcripts affected by Treatment

We used the R software environment and a mixed linear model ANOVA with the Maanova package for analyzing the expression data (Kerr, et al., 2000, Kerr and Churchill, 2001, Wolfinger, et al., 2001). Raw data were compiled as expression values using the RMA (Robust Multichip Average) expression measure for Affymetrix GeneChip® arrays (Bolstad, et al., 2003). Data were run in a series of 2 iterations, thus batch effects were accounted for with Batch Model Statistics. Our analysis focused on an ANOVA model testing for a fixed effect of the treatment (control, 0.1 mg/kg, 1mg/kg, or 10 mg/kg).

Although false-discovery rates (FDR) were calculated from permutation based P-values using the q-value software implemented in the R statistical package (Storey and Tibshirani, 2003), no significantly differentially expressed genes were detected under a reasonable threshold. Because of this, transcript expression results were analyzed via ANOVA using the Fs statistic. We tested for significance of the treatment with distribution free significance levels obtained through 1000 permutations of the data, shuffling biological samples to generate the distribution of the test statistic under the null hypothesis of no differential expression among treatments.

These statistics resulted in 1513 gene transcripts with $p < 0.05$. All genes with a p-value < 0.01 were tabulated for analysis. Genes with $p < 0.01$ of known identity are given in Table 9. Of the transcripts with the lowest p-values, suggesting the strongest effects of PCB treatment, a hypothesis-driven list of 27 genes were selected for further statistical scrutiny and of these 6 were selected as suggested candidates for follow-up study via real time PCR. Expression values of those genes were submitted to ANOVA, for parametrically distributed data, or permutations analysis for skewed, non-normal data. A Tukey-Kramer post-hoc comparison was used to determine significant differences between treatment groups following a significant treatment effect (Table 10). This approach increases statistical power by requiring post-hoc p-value correction for a sublist of only 27 genes, instead of a correction for 30,000 tests as was required for analyzing the entire GeneChip. It furthermore allowed more accurate selection of 6 follow-up genes for real time PCR.

Functional Group Analysis

Gene transcripts found to be most affected by A1221 exposure were categorized according to gene product function to infer whether any

functional groups were strongly represented in the results. The top 405 genes, with a p-value < 0.01, were submitted to Gene Ontology (GOfunctional grouping analysis using Multi-Protein Survey System (MPSS) online (http://www.scbit.org/mpss/) (Hao, et al., 2005). GO charts are a standardized and widely accepted method of representation for classifying a large list of genes according to the function of the protein products. Results were organized into the three primary GO categories of Cellular Component (Table 11), Biological Process (Table 12), and Molecular Function (Table 13). Many of the 405 genes with p < 0.01 have no known function, and several of them are non-identical transcripts of the same protein. Because of this, only 98 genes were recognized by the MPSS software, of which 50, 66, and 82 were matched within each of the primary GO categories, respectively. In addition, GO terminology permits unique proteins to be assigned to multiple functional categories.

To supplement the GO results and to provide a tissue-specific analysis of affected genes, we additionally applied literature research to each significant Affymetrix transcript (p < 0.01) to determine its gene identity and the function of its protein product. Information was collected from any or all of the following sources, depending on the availability of information for each particular gene: the NetAffx web search engine (http://www.affymetrix.com/analysis/index.affx), BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) searches of transcript sequences, and/or RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) searches for highly homologous genes in other species (> 98.5 % homology). In addition, PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) literature searches were conducted to accurately determine functional roles of each protein product within the brain, and Information Hyperlinked Over Proteins (ihop)

(http://www.ihop-net.org/UniPub/iHOP/) and Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov.content.lib.utexas.edu:2048/entrez/query.fcgi?db=OMIM) databases were consulted for additional, cross-species information. In the instances where a protein product has multiple functions dependent upon source tissue, only brain-specific functions were recorded because all RNA originated in the preoptic area of the brain. In total, 48% of transcripts significantly affected by treatment have no known identity or function, including proteins of known function that are not currently understood to be expressed within the brain. Each transcript was assigned to only one category. These results were then organized into broad gene functional categories that may be viewed in Table 14.

Transcriptional Response Element Analysis

Transcriptional response elements (TREs) for transcripts with $p < 0.01$ were extracted via Gene2Promoter software release 4.3 (Genomatix Software GmbH) (Cartharius, et al., 2005, Kramer-Hammerle, et al., 2005, Quandt, et al., 1995) using recommended Genomatix-prescribed promoter sequence length. Genes were submitted for analysis in groups of five using Affymetrix ID or selected gene search terms, and results were subsequently compiled. The most significant 50 genes were submitted because 50 is the recommended maximum number to include in a single comparative analysis (Genomatix Software GmbH guidelines). The average numbers of TRE repeats per gene are plotted in Figure 16 and TREs common to the greatest percentage of genes are given in Figure 15.

Cluster Analysis

Clustering analysis provides a non-subjective approach to grouping data by gene expression profile. This method is especially useful for assessing relative frequencies of dose-response curves when non-linear relationships are expected, as with U-shaped or inverted U-shaped dose-response curves that often accompany endocrine disrupting chemicals in toxicological research (Weltje, et al., 2005). Adaptive Quality-Based Clustering software (De Smet, et al., 2002) was used to generate dose-response clusters across treatment groups of genes with a p-value < 0.01, with the following parameters: 60% probability of genes belonging to a cluster, and a minimum of 4 genes allowed per cluster. Results are presented in Figure 17.

Results

AFFECTED GENES

ANOVA results revealed 1512 transcripts with a nominal p-value < 0.05, and 405 transcripts having a nominal p-value < 0.01, of which 215 are non-redundant genes of known identity. The list of genes with $p < 0.01$ was tabulated and referenced for further analysis. The number of genes from this list were grouped according to chromosomal location, and normalized to the overall number of genes on the microarray belonging to the same chromosome. No bias was observed in the chromosomal location of affected genes (3.5 to 6.7 % of genes on each chromosome were represented). Identifiable genes are listed in Table 9, grouped categorically by p-value.

Table 9. Transcripts Used in Analysis. Transcripts with no assigned name (ESTs or unknown genes) and multiple transcripts from the same gene are not included in this list, leaving 215 genes in Table 9. These are grouped by p-value into three sections : $p < 0.0005$, $0.0005 < p < 0.005$, and $0.005 < p < 0.01$.

Table 9: Affected Transcripts (nominal $p < 0.01$)

Nominal p < 0.0005
hemoglobin beta chain complex **(appears twice)**
similar to heterogeneous nuclear ribonucleoprotein G – human
hemoglobin alpha, adult chain 1 or alpha 2 chain **(appears twice)**
glucocorticoid modulatory element binding protein 2
C1q and tumor necrosis factor related protein 4 (predicted
Similar to NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 (predicted)
MAM domain containing glycosylphosphatidylinositol anchor 1 (predicted)
cytochrome P450, family 7, subfamily a, polypeptide 1
similar to Unconventional myosin-9b /// similar to spermatogenesis associated glutamate (E)-rich p
lumican
T-box 2 (predicted)
cathepsin L
opioid binding protein/cell adhesion molecule-like
similar to inositol 1,3,4-triphosphate 5/6 kinase
F-box only protein 32
Churchill domain containing 1 (predicted)
gap junction membrane channel protein alpha 5

Table 9 continues on the following page

Table 9 Continued

Nominal p-value: 0.0005 < p < 0.005	
Kruppel-like factor 12 (predicted)	similar to C11orf17 protein (predicted)
Similar to breast cancer membrane protein 101	CD5 antigen-like
phospholipase A2, group IIA (platelets, synovial fluid)	insulin receptor-related receptor
talin 1	upstream transcription factor 2
mitogen-activated protein kinase 15	prohibitin 2
Crystallin, gamma S	XK-related protein 5
similar to CREBBP/EP300 inhibitory protein 1	ubiquitin D
intraflagellar transport 74 homolog (Chlamydomonas)	aminolevulinic acid synthase 2
extracellular link domain-containing 1 (predicted)	RT1 class Ib, locus S3
solute carrier family 22 (organic cation transporter), member 2	ADP-ribosyltransferase 5
stimulated by retinoic acid gene 6 homolog (mouse)	cut-like 2 (Drosophila) (predicted)
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) (predicted) (appears twice)	selenium binding protein 2
Enthoprotin	neuregulin 1
Similar to [Ascaris lumbricoides mRNA sequence.]	osteoglycin (predicted)
FK506 binding protein 9	syntaxin binding protein 1
reproductive homeobox on X chromosome, 9	Ankyrin 3, epithelial
integrin beta 3 binding protein (beta3-endonexin)	triadin
reproductive homeobox on X chromosome, 9	splicing factor 3a, subunit 1 (predicted)
nicotinamide N-methyltransferase (predicted)	similar to C14orf25 protein (predicted)
gap junction membrane channel protein beta 3	Syntaxin 8
Cyclin M1 (predicted)	2,3-bisphosphoglycerate mutase
nudix (nucleotide diphosphate linked moiety X)-type motif 3	casein kinase II, alpha 1 polypeptide
SH2 domain binding protein 1 (tetratricopeptide repeat containing)	chloride channel K1
mitogen-activated protein kinase 8 interacting protein 3	guanine nucleotide binding protein, alpha 14
G protein-coupled receptor 39	ADP-ribosyltransferase 1 (predicted)
Similar to Zinc finger protein 184 (predicted)	Notch gene homolog 2 (Drosophila)
Low density lipoprotein receptor-related protein 1	vimentin
Retinoic acid induced 1 (predicted)	similar to C11orf17 protein (predicted)
insulin-like growth factor binding protein 7	C-reactive protein, pentraxin-related
v-ets erythroblastosis virus E26 oncogene like (avian)	adenylate cyclase 6
cytochrome P450, subfamily 2G, polypeptide 1	Insulin related protein 2 (islet 2)
Similar to neurobeachin (predicted)	CD24 antigen
Similar to WAC (predicted)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19
melanocortin 5 receptor	Corticotropin releasing hormone receptor 2
kinesin light chain 3	similar to Ras-related protein Rab-1B
laminin, alpha 2 (predicted)	Upstream binding protein 1 (predicted)
complement component 1, q subcomponent, gamma polypeptide	similar to cDNA sequence BC020002
glutamyl aminopeptidase	homer homolog 1 (Drosophila)
Prolylcarboxypeptidase (angiotensinase C) (predicted)	Unknown (protein for MGC:72614)
Smg-7 homolog, nonsense mediated mRNA decay factor	Noggin
natriuretic peptide precursor type C	Similar to nemo like kinase (predicted)
Hairy/enhancer-of-split related with YRPW motif-like	Grainyhead-like 1 (Drosophila) (predicted)
AHNAK nucleoprotein (desmoyokin)	Zinc finger protein 503 (predicted)
Chondroitin sulfate proteoglycan 4	tocopherol (alpha) transfer protein
thyroid hormone receptor associated protein 1 (predicted)	nuclear receptor coactivator 6
CCAAT/enhancer binding protein , epsilon	glutathione S-transferase A3
chemokine (C-C motif) ligand 22	osteoglycin (predicted)
interferon induced transmembrane protein 1 (predicted)	Similar to AP2 associated kinase 1 (predicted)
SAM domain, SH3 domain and nuclear localization signals, 1	similar to ovostatin-2 (predicted)
interleukin 2 receptor, gamma (severe combined immuno-deficiency)	protein kinase C, alpha
gap junction membrane channel protein beta 2	PRKC, apoptosis, WT1, regulator
cyclin-dependent kinase (CDC2-like) 10	

Table 9 continues on the following page

Table 9 Continued

Nominal p-value: 0.005 < p < 0.01	
potassium inwardly-rectifying channel, subfamily J, member 5	Solute carrier family 9, member 1
adrenergic receptor, alpha 2b	neurobeachin-like 2 (predicted)
paired-like homeodomain transcription factor 1	interleukin 24
PREDICTED: similar to lipocalin-interacting membrane receptor	ciliary neurotrophic factor
gastric inhibitory polypeptide	neuregulin 2
X transporter protein 3	annexin A1
hydroxysteroid (17-beta) dehydrogenase 9	ribosomal protein L17
similar to brain carcinoembryonic antigen	pregnancy-induced growth inhibitor
Anterior pharynx defective 1b homolog (C. elegans)	ribonucleotide reductase M1 (mapped)
PREDICTED: similar to PHD finger protein 20-like 1 isoform1	fibrinogen, alpha polypeptide
coatomer protein complex, subunit beta 2 (beta prime)	melanoma cell adhesion molecule
proteosome (prosome, macropain) subunit, beta type 9	allograft inflammatory factor 1
zinc finger protein 213 (predicted)	Cyclin G2 (predicted)
beta heavy chain of outer-arm axonemal dynein ATPase	defensin beta 1
similar to replication protein-binding trans-activator RBT1	chemokine (C-C motif) ligand 4
G protein-coupled receptor 23 (predicted)	fibroblast growth factor 21
Mitochondrial tumor suppressor 1	interferon beta 1, fibroblast
Centaurin, beta 5 (predicted)	troponin I type 3 (cardiac)
Toll interacting protein (predicted)	Reticulocalbin 1 (predicted)
zinc finger, FYVE domain containing 9 (predicted)	Spondin 1
citrate lyase beta like	Complement component 3
Deiodinase, iodothyronine, type II	crystallin, beta B3
similar to mKIAA0159 protein (predicted)	zinc finger protein 96
Similar to RNA (guanine-9-) methyltransferase domain containing 2	valyl-tRNA synthetase 2
Moloney leukemia virus 10-like 1 (predicted)	secretin
potassium inwardly-rectifying channel, subfamily J, member 3	paraoxonase 1
nudix (nucleoside diphosphate linked moiety X)-type motif 6	cathepsin W
procollagen, type VI, alpha 3 (predicted)	ras homolog gene family, member V
calcium channel, voltage-dependent, L type, alpha 1C subunit	transgelin 2
Similar to microtubule associated serine/threonine kinase 2	RT1 class I, CE16
polymerase (RNA) II (DNA directed) polypeptide H (predicted)	Pbx/knotted 1 homeobox
Similar to RAP2A, member of RAS oncogene family (predicted)	Similar to Ab2-095
ring finger protein 190	Roundabout homolog 2 (Drosophila)
myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11	Similar to Protein C20orf129 homolog
nsulin responsive sequence DNA binding protein-1	Similar to IQ motif and WD repeats 1
Regulator of nonsense transcripts 1 (predicted)	myosin IG
DNA cross-link repair 1B, PSO2 homolog (S. cerevisiae)	similar to KIAA0339 protein
phospholipase A2, group IVA (cytosolic, calcium-dependent)	Phospholipase C, beta 4
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	vacuolar protein sorting 37C (predicted)
solute carrier family 39 (iron-regulated transporter), member 1	interleukin 17B
purinergic receptor P2X, ligand-gated ion channel, 5	regulator of G-protein signaling 6
RAB5B, member RAS oncogene family (predicted)	nephroblastoma overexpressed gene
McKusick-Kaufman syndrome protein	secretoglobin, family 3A, member 1

CANDIDATE GENES

Because of previously documented effects of A1221 exposure on female reproductive behaviors and fertility, the authors hypothesize an endocrine-disruption-mediated mechanism. Predicting that the genes most strongly affected by PCB exposure would be hormone and neurotransmitter-related genes common to the preoptic area, we extracted a list of 27 genes suspected of being involved in PCB-mediated altered transcription from amongst the genes with a nominal p-value < 0.05. These genes, shown in Table 10, were selected based on documented PCB effects *in vivo* and *in vitro*. Transcript expression levels were submitted to Tukey-Kramer post-hoc analysis to determine possible significant differences between treatment groups. Significant differences between treated groups and control are indicated in the final column of Table 10.

Table 10: Candidate Gene List

27 candidate genes were selected from the list of genes with a p-value < 0.01. These genes underwent Tukey-Kramer post-hoc analysis to determine significant differences between groups. Genes found to have a significant difference between a treatment group and control ($p < 0.05$), and the direction of the effect, are indicated in the final column. Results are shown in order of nominal p-value.

Nominal p-value	Affymetrix ID	Gene ID	Significant Post-hoc Comparison with Control ($p < 0.05$)
0.019	1381606_at	stimulated by retinoic acid gene 6 homolog	
0.001	1382202_at	cut-like 2 (Drosophila) (predicted)	
0.003	1371142_at	cytochrome P450, subfamily 2G, polypeptide 1	
0.004	1389931_at	corticotropin releasing hormone receptor 2	1 mg/kg < Control
0.004	1372176_at	protein kinase C, alpha	
0.005	1371072_at	nuclear receptor coactivator 6	1 mg/kg < Control
0.005	1386156_at	thyroid hormone receptor associated protein 1	1 mg/kg > Control
0.005	1370567_at	adrenergic receptor, alpha 2b	
0.005	1369898_a_at	gastric inhibitory polypeptide	
0.005	1387994_at	hydroxysteroid (17-beta) dehydrogenase 9	1 & 10 mg/kg < Control
0.007	1391033_s_at	neuregulin 2	
0.011	1369908_at	corticotropin releasing hormone binding protein	1 & 10 mg/kg < Control
0.011	1381449_s_at	transforming growth factor alpha	
0.015	1370566_at	retinol dehydrogenase 2	
0.016	1369533_a_at	5-hydroxytryptamine (serotonin) receptor 4	0.1 mg/kg < Control
0.024	1384868_at	estrogen receptor alpha	10 mg/kg > Control
0.024	1388260_a_at	leptin receptor	
0.025	1398289_a_at	corticotropin releasing hormone receptor 1	0.1 mg/kg < Control
0.030	1388009_at	thyrotropin releasing hormone receptor	10 mg/kg > Control
0.030	1367851_at	prostaglandin D2 synthase	
0.036	1371762_at	retinol binding protein 4, plasma	
0.037	1368285_at	sex hormone binding globulin	
0.038	1369760_a_at	estrogen receptor beta	10 mg/kg > Control
0.038	1388239_at	period homolog 3	
0.038	1382916_at	thyroid hormone receptor beta	
0.041	1371120_s_at	bradykinin receptor, beta 2	
0.043	1375720_at	gamma-aminobutyric acid (GABA) B receptor 1	

In addition to those effects, several significant differences between treated groups were observed (Figure 14). Specifically, for estrogen receptor alpha (ER-alpha) and beta (ER-beta) the 10 mg/kg group had significantly higher expression than the 1 mg/kg group (Figure 14B). The 1 mg/kg group had significantly higher expression than the 10 mg/kg group for thyroid hormone receptor associated protein 1 (Figure 14A). The 10 mg/kg group had significantly higher expression than the 0.1 or 1 mg/kg groups for "stimulated by retinoic acid gene 6 homolog" and the 10 mg/kg group had higher expression than the 0.1 mg/kg group for retinol dehydrogenase 2. The 10 mg/kg also had significantly higher expression of corticotropin releasing hormone receptor 1 (CRHR1) whereas the 1 mg/kg group had significantly lower expression of CRHR2 (Figure 14C) and nuclear receptor coactivator 6 than all other groups.

Figure 14: Expression profiles for estrogen-, corticotropin releasing hormone- and thyroid hormone-related genes

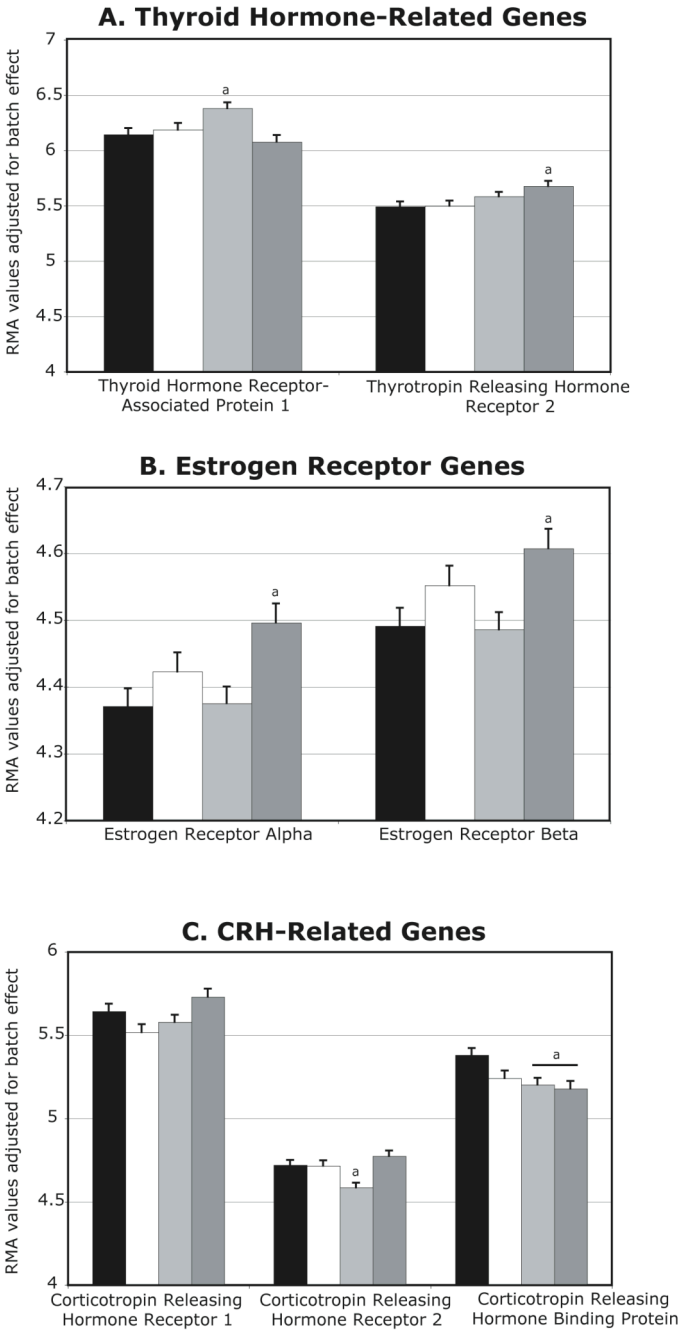


Figure 14: RMA results in $\log_2$ format were adjusted for batch effect and gene expression profiles are plotted here. Magnitude of expression differences may appear smaller here due to transformation of the data. A. Thyroid hormone-related genes. B. Estrogen receptors alpha and beta. C. Corticotropin releasing hormone-related genes.
a. $p < 0.05$ vs. Control (significant differences between treated groups are not shown)

GENE ONTOLOGY (GO) FUNCTIONAL GROUP ANALYSIS

SwisProt identifiers provided by Affymetrix annotation files for the list of genes with a p-value < 0.01 were submitted to GeneOntology (GO) analysis via Multi-Protein Service System (MPSS) algorithms. One GO tree was generated for each of three overall categories: Cellular Component (Table 11), Biological Process (Table 12), and Molecular Function (Table 13). A total of 98 proteins were recognized from the list of 405 genes ($p < 0.01$), which included 215 non-redundant identified genes. Of these 98 proteins, 50 were associated with a cellular component, 66 with a biological process, and 82 with a molecular function.

Of the 50 gene products with documented cellular location, 23 are localized to a membrane, and 24 are intracellular. The membrane proteins are best described as integral to the plasma membrane. Intracellularly located proteins are found primarily in the cytoplasm (16 of 24 gene products), and secondarily in the nucleus (10 of 24 gene products).

Table 11. Gene Ontology (GO) results: **Cellular Component** (50 genes matched)

The number of gene products per category are given in parentheses. Gene products may be assigned multiple functions.

1) Unlocalized (2)
   - A) Protein kinase CK2 complex (1)
   - B) Ribonucleoside-diphosphate reductase complex (1)
2) Extracellular (5)
   - A) Extracellular space (1)
3) Cell (44)
   - A) Membrane (23)
     - i) Plasma Membrane (10)
     - ii) Endomembrane system (2)
     - iii) Extrinsic to membrane (1)
     - iv) Inner membrane (1)
     - v) Integral to membrane (11)
     - vi) Mitochondrial membrane (1)
   - B) Cell Body (1)
   - C) Cell Fraction (2)
   - D) Cell projection (1)
     - i) Neuronal cell projection (1)
   - E) Cell surface (2)
   - F) Intracellular (24)
     - i) Ubiquitin ligase complex (1)
     - ii) Nucleus (10)
       - (1) Nucleoplasm (1)
       - (2) Proteasome complex (sensu Eukarya) (1)
     - iii) Cytoplasm (16)
       - (1) Sarcoplasm (1)
       - (2) Vacuole (1)
       - (3) Muscle fiber (1)
       - (4) Golgi apparatus (2)
       - (5) Mitochondrion (1)
       - (6) Cytoskeleton (4)
       - (7) Cytosol (3)
       - (8) Endoplasmic reticulum (2)
       - (9) Citrate lyase complex (1)
       - (10) Cytoplasmic ubiquitin ligase complex (1)
       - (11) Cytoplasmic vesicle (1)

In the Biological Process category, most of the affected gene products (54 of 66) are assigned a physiological process role, primarily in cellular metabolism (28 of 54 gene products). The majority of remaining gene products are split evenly between response to stress or external stimuli, or development (primarily morphogenesis) (13 of 54 gene products, each). Results are given in Table 12.

Table 12. Gene Ontology (GO) results: **Biological Process** (66 Genes matched)

The number of gene products per category are given in parentheses. Gene products may be assigned multiple functions.

1) Regulation of biological process (15)
   - A) Regulation of enzyme activity (1)
     - i) Positive regulation of enzyme activity
   - B) Regulation of physiological process (8)
     - i) Regulation of secretion (1)
     - ii) Regulation of vascular permeability (1)
     - iii) Regulation of metabolism (6)
   - C) Regulation of development (5)
     - i) Regulation of growth (3)
     - ii) Regulation of cell differentiation (2)
   - D) Regulation of cellular process (7)
     - i) Regulation of cell proliferation (4)
     - ii) Regulation of cell size (4)
     - iii) Regulation of cell volume (1)
     - iv) Regulation of membrane potential (1)
     - v) Regulation of programmed cell death (2)
     - vi) Regulation of growth (3)
     - vii) Regulation of cell differentiation (2)
     - viii) Regulation of signal transduction (2)
2) Physiological process (54)
   - A) Secretion (3)
     - i) Regulation of secretion (1)
     - ii) Fluid secretion (1)
     - iii) Hormone secretion (2)
   - B) Regulation of physiological process (8)
     - i) Regulation of secretion (1)
     - ii) Regulation of vascular permeability (1)
     - iii) Regulation of metabolism (6)
   - C) Response to stimulus (13)
     - i) Response to stress (7)
     - ii) Response to external stimulus (12)
     - iii) Response to endogenous stimulus (1)
   - D) Organismal physiological process (7)
     - i) Regulation of body fluids (2)
     - ii) Circulation (1)
     - iii) Excretion (1)
     - iv) Immune response (5)
   - E) Coagulation (1)
     - i) Blood coagulation (1)
   - F) Death (2)
     - i) Cell death (2)
   - G) Homeostasis (2)
     - i) Ion homeostasis (1)
     - ii) Cell homeostasis (2)

iii) *(Table 12 continues on the following page)*

Table 12 Continued

- H) Metabolism (28)
  - i) Regulation of metabolism (6)
  - ii) Protein metabolism (12)
  - iii) Organic acid metabolism (2)
  - iv) Oxygen and reactive oxygen species metabolism (1)
  - v) Phosphorus metabolism (4)
  - vi) Nucleobase, nucleoside, nucleotide and nucleic acid metabolism (8)
  - vii) Energy pathways (1)
  - viii) Hormone metabolism (2)
  - ix) Lipid metabolism (5)
  - x) Catabolism (6)
  - xi) Carbohydrate metabolism (1)
  - xii) Biosynthesis (3)
  - xiii) Aromatic compound metabolism (1)
  - xiv) Amino acid derivative metabolism (1)
  - xv) Amine metabolism (1)
  - xvi) Alcohol metabolism (1)
- I) Cellular physiological process (17)
  - i) Cell motility (2)
  - ii) Cell growth and/or maintenance (15)
  - iii) Cell death (2)

3) Development (13)
- A) Regulation of development (5)
  - i) Regulation of growth (3)
  - ii) Regulation of cell differentiation (2)
- B) Morphogenesis (11)
  - i) Organogenesis (7)
  - ii) Appendage morphogenesis (1)
  - iii) Embryonic morphogenesis (1)
  - iv) Cellular morphogenesis (4)
- C) Growth (6)
  - i) Regulation of growth (3)
  - ii) Tissue regeneration (2)
  - iii) Cell growth (4)
- D) Embryonic development (1)
  - i) Embryonic morphogenesis (1)
- E) Cell differentiation (4)
  - i) Neuron differentiation (2)
  - ii) Regulation of cell differentiation (2)
  - iii) Myeloid blood cell differentiation (1)
- F) Cell fate commitment (1)
  - i) Cell fate determination (1)

4) Cellular process (31)
- A) Regulation of cellular process (7)
  - i) Regulation of cell proliferation (4)
  - ii) Regulation of cell size (4)
  - iii) Regulation of cell volume (1)
  - iv) Regulation of membrane potential (1)
  - v) Regulation of programmed cell death (2)
  - vi) Regulation of cell growth (3)
  - vii) Regulation of cell differentiation (2)
  - viii) Regulation of signal transduction (2)

ix) *Table 12 continues on the following page*

Table 12 Continued

- B) Cellular physiological process (17)
  - i) Cell motility (2)
  - ii) Cell growth and/or maintenance (15)
  - iii) Cell death (2)
- C) Cell differentiation (4)
  - i) Neuron differentiation (2)
  - ii) Regulation of cell differentiation (2)
  - iii) Myeloid blood cell differentiation (1)
  - iv) Cell fate commitment (1)
- D) Cell communication (16)
  - i) Signal transduction (11)
  - ii) Cell-cell signaling (4)
  - iii) Cell adhesion (3)

5) Behavior (1)
   - A) Feeding behavior (1)

In the Molecular Function category, 44 of 82 gene products are implicated in binding, most of which bind proteins, nucleotides, and nucleic acids. The second most common category is catalytically active gene products, primarily hydrolases (29 of 82), and finally 19 of 82 gene products are involved in signal transduction (again, mostly hydrolase-based intracellular signal transduction). Results are shown in Table 13.

Table 13. Gene Ontology (GO) results: **Molecular Function** (82 Genes matched)

1) Structural molecule activity (4)
   A) Structural constituent of cytoskeleton (2)
   B) Structural constituent of eye lens (1)
2) Transcription regulator activity (7)
   A) RNA polymerase II transcription factor activity (1)
   B) Transcription cofactor activity (1)
   C) Transcription factor activity (5)
3) Transporter activity (8)
   A) Drug transporter activity (1)
   B) Ion transporter activity (2)
   C) Channel/pore class transporter activity (4)
   D) Carrier activity (2)
   E) Auxiliary transport protein activity (1)
   F) Amine/polyamine transporter activity (1)
4) Signal transducer activity (19)
   A) Receptor signaling protein activity (1)
   B) Receptor activity (9)
   C) Receptor binding (8)
5) Motor activity (1)
6) Enzyme regulator activity (1)
7) Catalytic activity (29)
   A) Transferase activity (8)
   B) Oxidoreductase activity (6)
   C) Lyase activity (1)
   D) Ligase activity (2)
   E) Kinase activity (4)
   F) Isomerase activity (1)
   G) Helicase activity (2)
   H) Hydrolase activity (12)
8) Binding (44)
   A) Selenium binding (1)
   B) Vitamin binding (1)
   C) Receptor binding (8)
   D) Nucleotide binding (10)
   E) Peptide binding (1)
   F) Protein binding (13)
   G) Lipid binding (1)
   H) Metal ion binding (3)
   I) Nucleic acid binding (10)
   J) Carbohydrate binding (1)
   K) Hormone binding (1)

CUSTOM FUNCTIONAL GROUP ANALYSIS

In addition to software-driven functional group derivation, we conducted a publications-based information compendium to determine likely tissue-specific roles for affected gene products ($p < 0.01$). Genes from this list were individually researched using published studies and reputable databases, and were categorized according to protein function. No identifying information (or no brain-specific role) was found for 189 transcripts (47.6%), which were labeled “unknown”. Of recognized transcripts, the most common functional groups are: blood-based proteins (13.9%), transcription factors (13.0%), intercellular signaling (11.5%) and intracellular signaling (11.1%). The hormone signaling functional category contained only 8.2% of recognized gene products. Results are presented in Table 14, along with a more detailed explanation of inclusive gene functions assigned to each category.

Table 14: Custom Functional Group Analysis

Literature searches and information from publicly available databases were used to generate custom brain-specific functional groups for the gene list with a p-value < 0.01 (n = 405 genes). Gene products were assigned to only a single functional group. Gene products with no known function or no known brain function are not included in this table. Results are given as number of genes per category or as % of known gene products from the list $p < 0.01$.

functional group	description	# genes	% known
toxin metabolism	xenobiotic degratory proteins	2	1.0%
cell metabolism	mitochondrial proteins	3	1.4%
cell cycle and DNA replication	DNA synthesis related proteins	10	4.8%
blood-brain barrier & brain vesicles	proteins associated with the blood-brain barrier or brain vesicle wall	5	2.4%
post-transcriptional modification	RNA modification and RNA editing proteins	5	2.4%
transcription	RNA synthesis related proteins	6	2.9%
translation	protein synthesis related proteins	7	3.4%
cytoskeletal element	gap junctions, cytoplasmic structural proteins, and anchoring proteins	9	4.3%
cell surface protein	extracellular matrix and cell adhesion proteins	10	4.8%
ion regulation	ion channels involved in neuronal excitability and cell homeostasis	10	4.8%
vesicle trafficking	proteins involved in endo- and exocytosis	10	4.8%
post-translational modification	ribosylation, sialyltransferase, and ubiquitination proteins as well as peptidases	11	5.3%
hormone signaling	hormones, receptors, and associated proteins	17	8.2%
intracellular signaling	phosphatases, kinases, phospholipases, and second messenger systems	23	11.1%
intercellular signaling	cytokines/growth factors and receptors, neurotransmitter receptors, and associated proteins	24	11.5%
transcription factor	Transcription factors and coregulatory proteins	27	13.0%
blood	Blood-specific cells & proteins, blood vessels, and Immune cells	29	13.9%
Total Known Genes		**209**	**100%**

TRANSCRIPTIONAL RESPONSE ELEMENT (TRE) ANALYSIS

Transcriptional response elements (TREs) for the 50 recognized transcripts with the lowest p-value were determined via Gene2Promoter, a software accessory in the Genomatix analysis package. Gene promoters were extracted by Gene2Promoter according to intrinsic software guidelines, and potential TREs were searched using likelihood probability matrices. Prediction of TRE functionality can be unreliable. Because we could not biochemically test the functionality of each recognized TRE, we refer to results as “potential TREs”.

The average number of potential TREs per gene were found by averaging the number of potential TRE sites (repeats) across all genes containing that TRE. Genes lacking a particular TRE in their promoter are not included in the average. Results are shown in Figure 15. Increased incidence of particular transcriptional response elements in the gene promoters of significantly affected genes may represent a transcriptional mechanism by which PCBs can alter gene transcription. The TRE occurring with the greatest frequency of repeats within affected genes is bound by the Nuclear Respiratory 1 Family of transcription factors (NRF1). GAGA box (GABF) TREs also occur in high frequency, and the Early Growth Factor/Nerve Growth Factor Induced Protein C family (EGRF) represents the third largest category. Following this TRE, the average frequency of repeats drops to a relatively uniform value.

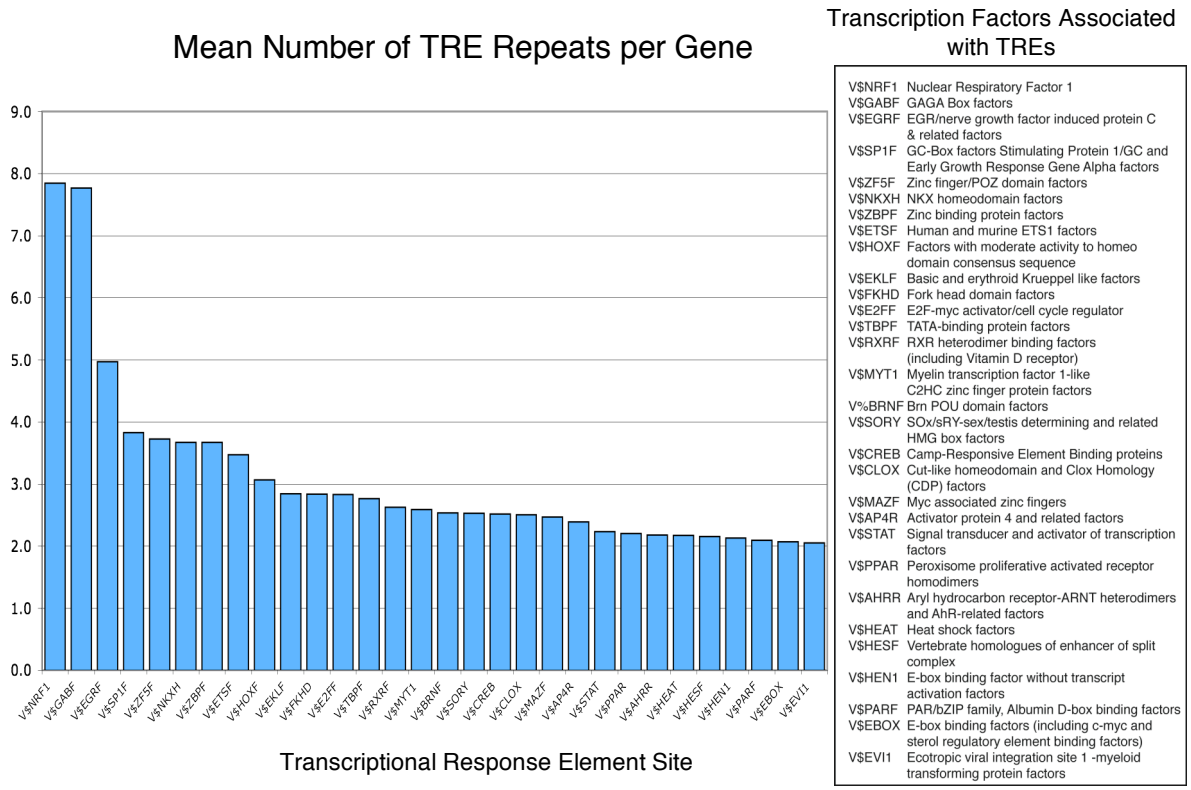


Figure 15. Transcriptional Response Element (TRE) analysis: Average # of TREs per Gene. Genomatix GmbH software Gene2Promoter was used to extract promoter sequences from the 50 recognized genes with the lowest p-values. TREs occurring with the greatest frequency per promoter are shown here (>2 per gene). TRE codes on the X-axis are given in the format of TRAFAC binding factor sites (http://trafac.cchmc.org/trafac/ShowFamily;jsessionid=9A36ACF574C8140AA38D0F8B4A44FA71).

Potential TREs common to the 50 most significant recognized genes are plotted in figure 16. The TRE most represented across the 50 tested genes was the Ets-1 family (Avian retroviral E-Twenty-Six), which includes Elk-1 (Ets-like gene 1) and Nuclear Respiratory Factor 2 (NRF2). Following this, the Homeobox Factor family with moderate activity (HOXF) is found in 90% of tested genes, and the mammalian equivalent of Drosophila Tinman, (NKXH) family of transcription factors is found in 84% of tested genes. Following these TREs come CREB, the ubiquitous cAMP-responsive binding protein site, the HEAT heat shock factors family TRE, and the SP1F TRE site, which is bound by Stimulating Proteins 1 and 2, TGF-beta inducible early gene (TIEG), and related factors.

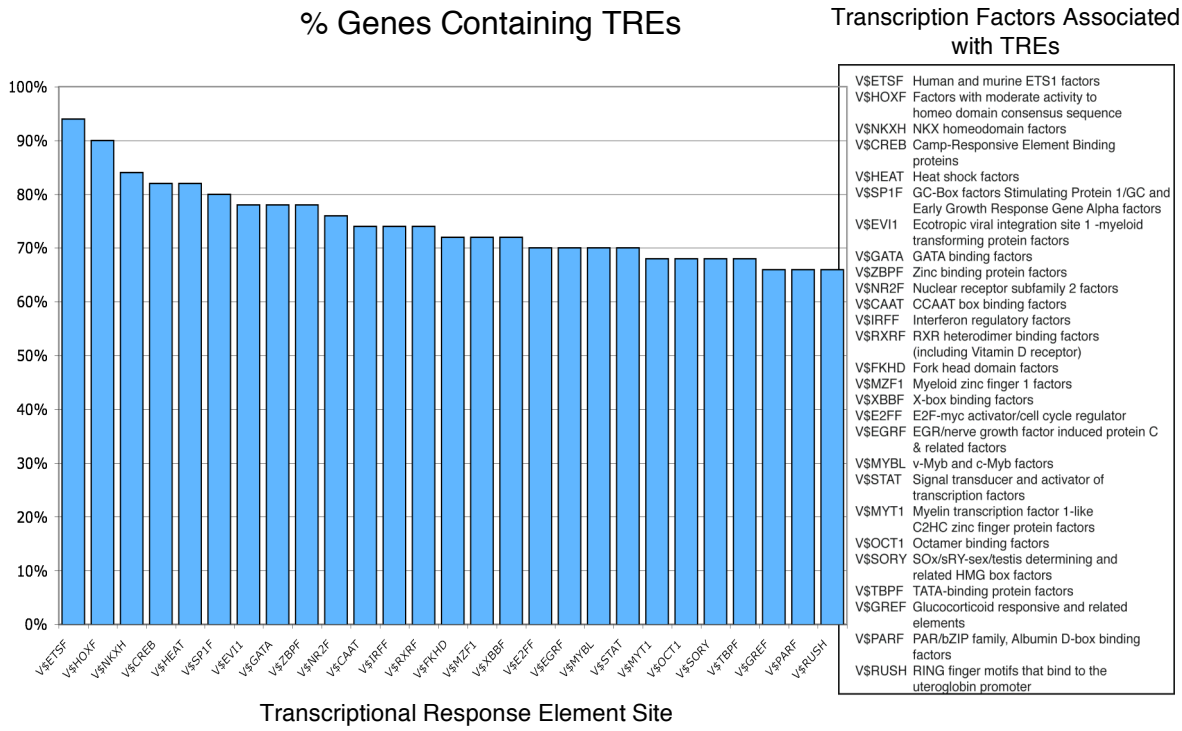


Figure 16. Transcriptional Response Element (TRE) analysis: % of Gene Promoters Containing Each TRE. Genomatix GmbH software Gene2Promoter was used to extract promoter sequences from the 50 recognized genes with the lowest p-values. The highest occurring TREs are shown here (those occurring in >65% of genes). TRE codes represent TRAFAC binding factor sites.

CLUSTER ANALYSIS

Dose-response curves of gene transcripts with a p-value < 0.01 were entered into cluster analysis using the Adaptive Quality-Based Clustering software (http://homes.esat.kuleuven.be/~thijs/Work/Clustering.html). Results are given in Figure 17. The criteria of 60% probability of fitting a cluster group, and a minimum of 4 transcripts per cluster were used. Transcripts were assigned to only one cluster, and dose-response curves that did not fit the clustering criteria are not included. Cluster analysis reveals commonalities between expression profiles but does not measure the statistical significance of differences between treatment groups. These results should be interpreted in the context of a metaanalysis examining overall trends in treatment effects within the data set.

Of the 405 gene transcripts submitted to analysis, 264 were placed within a cluster (~65%), and of these 128 transcripts (48%) were of unknown function. The 11 clusters resulting from analysis, randomly assigned a numerical ID, are arranged in Figure 17 according to shape: inverted U-shaped, zig-zag down, and U-shaped. No linear dose-response curves emerged as a significant cluster from the analysis parameters employed in this study.

In total, 48 transcripts had an inverted U-shaped dose-response profile, which is characterized by the 0.1 and 1 mg/kg PCB groups exhibiting increased expression and the 10 mg/kg group exhibiting equal or decreased expression compared with control. Unknown transcripts formed the largest portion of this group (n=24) with cell cycle genes forming the next largest group (n=5). 40 genes exhibited a zig-zag down curve, wherein the 1 mg/kg group showed some recovery from a general trend of depressed gene expression. The largest functional categories in the zig zag down group were unknown transcripts (n=15), followed by blood-related transcripts

(n=11), and blood-brain barrier-related transcripts (n=4). By far the largest group was the U-shaped dose-response curve. The majority of transcripts in this group (n=123) fell into Cluster 1, which was characterized by increased gene expression in the 10 mg/kg group (Figure 17). In this group, 88 transcripts were of unknown function, 14 were blood-related, and 13 were transcription factors.

Figure 17: Cluster Analysis Results

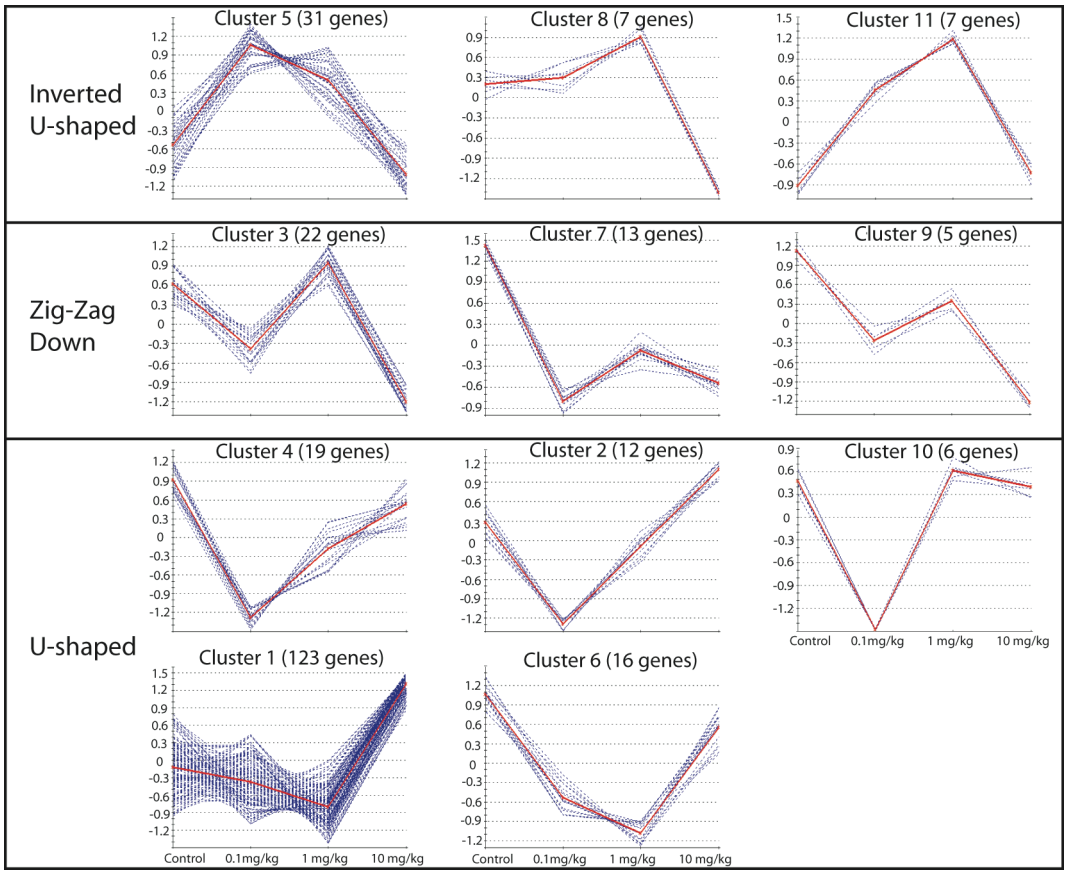


Figure 17. Cluster Analysis results for genes with a p-value < 0.01. Data were generated on Adaptive Quality-Based Clustering software. For a cluster membership probability of 60% and a minimum of 4 genes per cluster, 11 graphs were generated. These have been grouped according to dose-response curve shape: Inverted U-shaped, Zig-Zag Down, and U-shaped. Data from each transcript is shown as a blue dashed line, and averaged curves are given in a solid red line. The Y-axis is centered around a normalized mean of all transcript expression values included in the cluster, given as 0. Deviation from this overall mean is indicated by positive or negative deflections from the 0 mark. The four treatment groups of Control, 0.1, 1, and 10 mg/kg PCBs are represented, respectively, by the numbers 1-4 on the X-axis. Dose-response relationships that do not conform to a common cluster are not included in the results.

Discussion

The present study addressed the question of whether fetal PCB treatment caused any permanent alterations in gene expression that would be manifested in adulthood. We focused this analysis on the POA, a brain region that is important for reproductive physiology and behavior (Uphouse and Caldarola-Pastuszka, 1993, Wersinger, et al., 1993, Wheaton, et al., 1975). Because relatively low doses of PCB were administered and more than 55 days elapsed between treatment to the fetus and tissue collection at approximately 7-8 weeks of age, we anticipated subtle effects of PCB exposure on gene expression. In concert with our expectations, we viewed the whole genome microarray as a hypothesis-generating tool to explore broad changes in patterns of gene expression influenced by environmental toxicant exposure. The large number of genes assayed on the chip generates a substantial amount of background noise against which a clear signal is difficult to determine. In addition, the frequency of false positive results varies directly with the number of tests, and thus the desired level of significance should be corrected by the approximately 30,000 transcripts assayed.

Given the conservative nature of statistical correction, the whole genome Biochip is not the appropriate tool for stating with quantitative certainty which genes have altered expression following intoxication. Instead, it provides a valuable service in broadening our investigation of PCB effects above and beyond our hypothesis of primarily endocrine-related effects, thereby highlighting future directions in which to channel toxicological research. In PCB toxicology, many of the molecular mechanisms of action are unknown. Here, we present results identifying the most differentially expressed genes in response to three different doses of PCB treatment. We

believe that studies of this nature provide novel insight into the global mechanisms of PCB influences on gene expression.

DIFFERENTIALLY AFFECTED GENES

A close look at the genes with the most affected gene expression profiles (those with the lowest p-values, $p < 0.01$) across treatment groups can present a more detailed picture of the effects of prenatal toxicant exposure. Aroclor 1221 and its metabolic by-products are well-documented endocrine-disrupting chemicals with multiple effects on estrogen signaling, including binding the estrogen receptor (Shekhar, et al., 1997) and initiating transcription of estrogen-responsive genes (Layton, et al., 2002), inhibiting the estrogen synthetic enzyme aromatase (Woodhouse and Cooke, 2004), and interfering with estrogen receptor beta gene expression (Salama, et al., 2003). Upon close examination, we observed a number of genes involved in the estrogen signaling pathway. Namely, MapK15/ERK8, “similar to breast cancer membrane protein 101”, “similar to breast cancer associated oncogene”/BCA3, “secretoglobin family 3A member 1”/HIN1, OKL38 and mac25 showed altered expression following PCB exposure. Many of these genes, possibly because of their estrogen sensitivity, are associated with breast cancer and development, although we cannot infer that their expression is similarly affected in mammary tissues as our current analyses were conducted in the POA.

PCB exposure is associated with disruption of the hypothalamic-pituitary-adrenal (HPA) axis, and several corticosterone releasing hormone-related genes were found to have altered expression levels following prenatal A1221 exposure. Methylsulfonyl PCB metabolites have been found to alter corticosterone synthesis (Johansson, et al., 1998a), and bind to the

glucocorticoid receptor (Johansson, et al., 1998b), and PCB exposure is linked with elevated basal corticosterone levels (Miller, et al., 1993).

Microarray results identified several HPA axis stress regulatory genes in the list of differentially expressed genes ($p < 0.01$), which may be attributed to HPA mediation by gonadal steroids in the preoptic area. The preoptic area is involved in the HPA axis, and is associated with stress response (Mccormick, et al., 2002, Viau, 2002), specifically with reference to signal initiation by the cortex/amygdala (Viau and Meaney, 1996), or signal intensity attenuation by sex steroid hormones (Herman and Cullinan, 1997, Mccormick, et al., 2002). In the HPA axis, corticotropin releasing hormone (CRH) in the hypothalamus (Cummings, et al., 1983) and preoptic area (Mcdonald, et al., 1994) induces release of adrenocorticotropic hormone (ACTH) from the pituitary, which then stimulates release of the "stress hormone" corticosterone from the adrenals; CRH also acts as a neuropeptide within the brain. One of the downregulated genes of interest in this study, CRH receptor 2 (CRHR2), is bound with high affinity by the endogenous ligands urocortin 2 and 3, and modulates stress response by maintaining ACTH release throughout the stressor and then terminating the stress response (Reyes, et al., 2001). It is thus believed to provide an anxiolytic effect counter to the anxiogenic properties of CRH receptor 1 (CRHR1) (Kishimoto, et al., 2000). CRHR2 ligand urocortin 3 mRNA positive nerve terminals project into the hypothalamus and nearby basal forebrain nuclei, and urocortin 3 protein expression in the brain was strongest in the median preoptic nucleus (Lewis, et al., 2001), which confirms colocalization of the ligand with its receptor CRHR2. Urocortin 3 expression is also rapidly upregulated in response to psychological stressors (Valdez, et al., 2004).

Another gene of interest was also downregulated: corticotropin releasing hormone binding protein (CRHBP) which is the only known binding

protein with high affinity for a neuropeptide, CRH (Jahn, et al., 2005). CRHBP is broadly expressed in the brain, including the hypothalamus and preoptic area (Cummings, et al., 1983). Approximately 50% of whole brain corticotropin releasing hormone (CRH) is bound by CRHBP, and thus made unavailable to bind to receptors (Behan, et al., 1995). CRHBP expression is mediated by gonadotropins in primate gonadal tissues (Xu, et al., 2005, Xu, et al., 2006), and thus may exhibit similar hormonal regulation in the brain. It has been suggested that CRHBP prevents generalized neuronal hyperexcitability of CRH (Aldenhoff, et al., 1983), in addition to its role in the HPA axis. This study presents the first documented evidence, to our knowledge, of a PCB effect on expression of corticotropin releasing hormone binding protein and corticotropin releasing hormone receptors 1 and 2.

Another notable gene that is affected by A1221 is ELAV-like 1 (HuR), a key player in post-translational mRNA stability. Previous results in our laboratory found altered post-transcriptional regulation following A1221 exposure to an immortalized GnRH cell line (Gore, et al., 2002). PCBs have also been shown to alter mRNA levels by increasing post-trancriptional mRNA stability of the steroidogenic enzymes CYP11B1 and CYP11B2 (Lin, et al., 2006a). The role that HuR plays in transcript stability may underlie some of the molecular effects of PCB exposure. In addition, its involvement in signal transduction and intracellular messenger cascades may correlate with the high number of disrupted intracellular signaling molecule genes, as discussed in functional group analysis.

Several other genes with low p-values are related to neurodegenerative and psychiatric diseases, although their roles in the POA are not well understood. Both neuregulin 1 and neuregulin 2, cytokines for which mutation or altered expression is related to a number of pathologies including Alzheimer's and schizophrenia susceptibility (Esper, et al., 2006,

Meeks, et al., 2006), were included amongst the most significantly affected genes. In addition, affected gene spondin 1 is known to interact with Alzheimer's-related protein amyloid beta precursor protein (APP) to inhibit its cleavage (Ho and Sudhof, 2004). To date, few studies have been conducted to specifically address the connection between environmental toxicant exposure and epidemiology, and thus only slight evidence exists for a role of PCBs in neurodegenerative diseases and dementia (Corrigan, et al., 2000, Landrigan, et al., 2005, Troster, et al., 1991). Although PCB exposure is believed to increase susceptibility to Parkinson's Disease (Caudle, et al., 2006), we did not observe any differential expression of genes related to Parkinson's, possibly because we focused on the POA.

Although our focus is on implications of altered expression of genes affected by PCB exposure ($p < 0.01$) we stress the necessity for follow-up procedures employing a more quantitative procedure. The interests of our laboratory lie in the documented effects of A1221 and other PCBs on female sexual development and behavior. Thus, we selected 27 candidate genes for additional analysis to determine which genes to study further with quantitative real time PCR. From the results given in Table 10, we chose the following genes showing significant post-hoc treatment effects vs. control, for quantitative real time PCR follow up: estrogen receptor alpha, estrogen receptor beta, corticotropin releasing hormone binding globulin, hydroxysteroid (17-beta) dehydrogenase, corticotropin releasing hormone receptor 2, and nuclear coactivator 6. In future studies beyond the scope of this dissertation, we will perform real-time PCR analyses of several of these candidate genes.

FUNCTIONAL GROUP ANALYSES

Overall, both the Gene Ontology and the custom functional group analyses were in agreement. Both methods revealed a preponderance of affected genes involved in transcriptional activity and intracellular signaling pathways, however, methodological differences between the two approaches highlighted several different findings as well. The GO approach revealed the large number of gene products residing in plasma membranes, and emphasized the strong presence of genes involved in signal transduction and response to external stimuli. The custom approach, however, provided a practical evaluation that revealed the involvement of blood and immune-related genes with the PCB-derived gene expression phenotype.

Because of the well-documented effects of A1221 on steroid hormone systems, we hypothesized that expression of primarily hormone- and neurotransmitter-related genes in the preoptic area would be most affected by exposure. However, functional group analysis did not reveal a large number of hormone-related genes. GeneOntology (GO) chart analysis suggests that the most common functional groups affected by PCB exposure are plasma membrane and cytoplasmic metabolic proteins, including hydrolase enzymes and nucleotide/nucleic acid binding proteins. Additionally, it is not surprising that PCBs disrupt so many membrane-related proteins, since their lipophilic nature allows them to pass through or reside in plasma membranes, thereby disrupting lipid bilayer fluidity (Tan, et al., 2004a). Possibly, developmental exposure leads to a permanent reactive altered expression of membrane-residing intrinsic proteins. Custom functional group analysis placed hormone-related genes at 8% of all results, the fifth largest group. These results imply that the most common long-lasting effects of prenatal PCB exposure on adult gene expression are in intracellular signaling pathways leading to transcriptional and post-

transcriptional control of gene expression. Although few endocrine-related genes seem to be affected by prenatal A1221 exposure, this does not undermine the important role of hormone-responsive gene products in the preoptic area.

Another common pathway affected in GO chart analysis involves receptor, protein, and nucleotide binding, such as for transcriptional activation, which may also present a proximal cause of adult gene expression disruption following prenatal PCB exposure. The majority of transcription factor transcripts identified in the list of significant genes ($p < 0.01$) are not well documented in scientific literature. Because of this, specific transcription factor-protein interactions and transcriptional response element binding sites cannot be determined. However, we speculate that because many transcription factors have proto-oncogenic capabilities, the altered expression we observed may be related to the increased risk of cancer faced by PCB-exposed individuals (Cope, et al., 2003, Ntp, 2006, Prince, et al., 2006). In addition, lightly chlorinated PCBs such as A1221 may correlate more strongly with breast cancer risk than the more heavily chlorinated congeners (Lucena, et al., 2001, Moysich, et al., 1998).

In the custom functional group analysis, the primary category of affected genes ($p < 0.01$) was blood-related (including alpha and beta hemoglobin, and Alas2). PCB exposure can decrease erythrocyte count and platelet volume, as well as lower immune response, and alter ratios of T helper and suppressor cells (Arnold, et al., 1993). PCBs can bind to hemoglobin directly (Rehulka and Minarik, 2004) which may lead to premature degradation. Altered levels of corticotropin releasing hormone-related genes may also be related to the affected expression of genes important in the immune system. Immunotoxic response is regulated partially by corticosterone, and abnormal expression of genes upstream of

corticosterone in PCB-treated groups could alter immune-related gene expression.

TRANSCRIPTIONAL RESPONSE ELEMENT (TRE) ANALYSIS

We conducted transcriptional response element (TRE) analysis on the list of significant genes ($p < 0.01$) in order to uncover possible DNA binding sites common to genes affected by PCB exposure. The results of this analysis suggest possible transcriptional mechanisms by which A1221 might be acting. Several genes that were strongly affected by PCB treatment, including CCAAT binding protein epsilon, and similar to CREBP inhibitory protein, may serve as a proximal mechanism for observed altered gene expression. Because we are unable to test the functionality of potential TREs statistically extracted from published promoter sequences, we cannot make definitive conclusions based on these results. Rather, we here examine possible contributions of the top ranking TREs to A1221-derived transcriptional influence. TREs found in > 65% of the top 50 recognized genes are discussed.

Results of TRE analysis fall into three general groups: blood and immune-specific transcription factor families, neurodevelopmental transcription factor families, and ubiquitous cell metabolic families. The TRE with the highest-ranking gene frequency, the ETS1-Factor (ETSF) plays a crucial role in pattern formation during development, mainly through its involvement in cell proliferation and differentiation, lymphoid cell development, angiogenesis, and apoptosis (Hallikas, et al., 2006, Lelievre, et al., 2001, Pei, et al., 2005). The strong representation of blood and immune-related functional groups in our data set of the top 50 affected transcripts could be related to A1221 influence at this site, or at another of the

blood/immune-related TREs, including EVI-1 (Ihle and Askew, 1989), GATA (Dai, et al., 2002), and NKXH (Nishida, et al., 2002).

Several neurodevelopmental TREs were identified from our analysis. The HoxF TRE is implicated in telencephalic brain patterning, most notably the associated transcription factors Phox2a/2b and Pitx3 which have powerful control over noradrenergic neuronal differentiation and tyrosine hydroxylase expression, respectively (Lebel, et al., 2001, Seo, et al., 2002). Effects of PCBs on decreased brain tissue content of norepinephrine are well documented (Meerts, et al., 2004c, Seegal, et al., 1985). Sp1 and Sp2 are common transcription factors for the SP1F TRE, found in cytochrome p450 steroidogenic enzyme genes, and vasopressin (Hammer, et al., 2004, Rabadan-Diehl, et al., 2000) and thereby associated with hormone signaling. CREB and HEAT are transcriptional binding element sequences that mediate cAMP and heat shock protein signaling, respectively. Cyclic AMP is activated ubiquitously in response to a number of intracellular signaling systems, and heat shock proteins mediate protein folding and degradation in many cell types. The high percentage of genes that include these transcriptional response elements is not surprising, considering their general commonality, however we do not rule out the possibility that A1221 may also target those transcriptional pathways. Finally, a putative estrogen response element was found in 42% of tested genes, and the putative androgen response element was found in 66% of tested genes.

CLUSTER ANALYSIS

Non-linear dose-response curves are indicative of steroid hormone action, whereby physiologically low or high concentrations elicit some response, and median concentrations have the opposite polarity of effect (Weltje, et al., 2005). As predicted, the preponderance of non-linear dose-

response curves within the data set of significant genes identified by microarrays ($p < 0.01$) suggests that prenatal A1221 is acting overall in a steroid-hormone or hormetic like mechanism. This would be expected if higher than normal levels of a hormonally active substance, such as PCBs, during a sensitive period of development resulted in desensitization or downregulation of downstream transcriptional targets. Possible mechanistic explanations for the prevalence of the U-shaped curve include: (1) direct binding of A1221 to steroid hormone receptors, (2) alteration of enzymatic activity related to steroid hormone formation or metabolism, (3) direct binding of A1221 to orphan nuclear receptors such as AhR, which may result in steroid hormone-like dose-response relationships, or (4) interference in intracellular signaling cascades in a hormone-rich region of the brain, which indirectly alters neuroendocrine levels of steroid hormones. Because of the many known disruptive modes of action for PCBs, it is likely that the observed alterations in gene transcription may be ascribed to several non-exclusive causative mechanisms, as these are not mutually exclusive events.

Many blood-related genes, including alpha and beta hemoglobin, fell into the dose-response curve described by cluster 7, showing depressed gene expression in all three treatment groups, suggesting either transcriptional coregulation or altered developmental patterning resulting in decreased vascularization in treated groups. Interestingly, immune-related genes such as fibrinogens, complement components, and leukocyte-specific chemokines, form most of the blood-related genes upregulated in the 10 mg/kg group (cluster 1), and this same treatment group exhibited decreased expression of blood-brain barrier-related genes (clusters 3 and 9). Because not only blood-related genes, but vascular epithelial and smooth-muscle genes had compromised transcript levels following PCB exposure, we believe that the most heuristic explanation is decreased vascularization of

the preoptic area in treated groups, although we acknowledge that multiple mechanisms most likely contribute to these observed effects. Steroid hormones, including estrogen, regulate blood vessel diameter and proliferation in the brain (Krause, et al., 2006), and PCBs have been shown to alter estrogen-mediated angiogenic signaling in the umbilical cord (Tavolari, et al., 2006). Although the effects of prenatal PCBs on brain vascularization have not yet been researched, diethylstilbestrol, an estrogenic toxicant, can alter estrogen-induced microvascularization in the pituitary (Pawllkowski, et al., 1996, Stehr-Green, et al., 1986).In addition to blood-related genes, of the transcripts categorized as transcription factors/cofactors, 3 were downregulated by PCB treatment, but 11 were upregulated. Similarly 4 transcripts in the intracellular signaling category were downregulated whereas 10 were upregulated.

Overall, cluster analysis confirmed the hypothesis that prenatal A1221 elicits non-linear dose-response curves in adult gene expression, suggesting that A1221 acts in a hormone-like manner. Whole genome microarray is particularly valuable for this method of analysis because it eliminates much of the bias involved in choosing genes to examine dose-response relationships.

Conclusion

After examining the effects of prenatal A1221 exposure on adult female gene expression in the preoptic area of the brain, we conclude that prenatal A1221 likely acts on adult gene expression mostly with a steroid hormone-typical non-linear dose response curve. Prenatal exposure to A1221 has greatest effects on blood and immune-related gene expression, and alters transcription factor mRNA levels. Putative steroid hormone receptor response elements were not as commonly localized to the most

strongly-affected genes, suggesting that the mechanisms for A1221's influence on gene expression probably lie in a complex interaction of disruptive effects at multiple levels of molecular interactions. The results of this study have illuminated many avenues for follow-up research investigating the connection between prenatal A1221 exposure and adult neuroendocrine gene expression. A1221 likely shares some mechanisms of action and patterns of molecular interaction with other endocrine-acting toxicants, for which prenatal exposure is a current concern for human health and wildlife survival. The present whole genome microarray study was a hypothesis-generating tool to benefit further research on this topic. The current findings may thus have a strong impact on further studies investigating environmental toxin exposure and disease susceptibility.

Overall Discussion

As persistent organic pollutants, PCBs represent an acknowledged health threat to humans and wildlife, and a warning against future development of industrial chemicals that have not been thoroughly tested in biological systems. The limits of PCB toxicity are not currently known, and every year new studies reveal previously unknown targets for PCB disruption. Novel findings are dependent on which PCB congener/mixture is used, the method of administration, the dose administered, the age at administration, the species under study, the endpoints evaluated, and which tissues or pathways are under scrutiny. It is becoming increasingly clear that in order to control for a high degree of variability in PCB studies, concatenated endpoints are necessary: all animals are treated identically (aside from a single variable), and multiple endpoints are measured from each litter. In this manner, information mined from diverse assays may be compared with confidence in order to achieve a clearer image of the pharmacological implications of PCB exposure.

To address these issues, this dissertation research examined the effects of low-dose PCB exposure during brain sexual development on female reproductive parameters and physiology. Namely, a range of doses (0, 0.1, 1 and 10 mg/kg) of the commercial PCB mixture A1221 was selected to mimic human and wildlife exposures. The selected endpoints include developmental, behavioral, physiological, and molecular data that offers a multi-phenotypic portrait of PCB disruption.

Developmental effects of PCBs on two generations of female rats

This dissertation research found that exposure of female rats to ecologically-relevant levels of A1221 during a sensitive period for brain sexual differentiation had long-term and unexpected results that extended into the second generation. Three possible sources of the observed multigenerational effects of PCB exposure are: altered maternal behaviors in the F1, epigenetic imprinting, or mutations to the germ cell genome or epigenome. Multigenerational effects on behavior and physiology have been observed in studies of maternal care, whereby natural variations in maternal care can alter expression of stress-related genes in the offspring, and can even modify maternal care *by* the offspring in adulthood (Meaney, 2001). This body of work serves as an example whereby maternal behaviors are the source of multigenerational transmission of a particular trait. Additionally, the effects of a single administration of beta-endorphin on peripheral immune serotonin production were measured in three generations of rats (Csaba, et al., 2005). As with the current study, the magnitude of effect increased with each generation following the exposed dam, and the authors attributed the mechanism to gene imprinting. A third explanation for multigenerational manifestation of the effects of toxin exposure can be found in epigenetic modification of germ line gametes. Studies examining exposure to vinclozalin, an anti-androgenic fungicide, found transmissible epigenetic alteration of the spermatocyte genome (Anway, et al., 2005, Chang, et al., 2006).

There are few studies examining the transmission of a single toxicological event treatment or experiential effect across multiple generations are rare compared with toxicological studies investigating chronic exposures across multiple generations. An elegant series of

experiments conducted by the MK Skinner laboratory investigating *in utero* vinclozalin exposure of male rats is a notable exception. This research group found the transmission of disease states or abnormalities in multiple organ groups for up to four generations (Anway, et al., 2006). Experiments documenting the effects of mouse prenatal exposure to diethylstilbestrol (DES), an estrogenic drug previously prescribed clinically as a miscarriage preventative, also found increased occurrence of gonadal tumors and lesions, and upregulation of estrogen-responsive gene transcription in multiple generations. The authors hypothesized that these effects were due to epigenetically altered methylation patterns on estrogen-sensitive genes, and possible germline mutations (Newbold, et al., 2006);

It is possible that the different generations following exposure might experience different magnitudes of effects. The research described in this dissertation did not directly investigate relative magnitude of effect in multiple generations because the F1 and F2 generation females were part of different experimental methodologies, and different endpoints were examined. Indeed, although data are presented for each generation in the DES and vinclozalin research discussed above, there have been no multigenerational transmission studies that statistically compare the magnitude of effect across multiple generations.

Whereas no gross morphological differences were observed in PCB-exposed pups, and no clear differences emerged from observations of developmental landmarks of eye opening and puberty onset in either the exposed or the second generation, altered steroid hormone levels were observed in the F2 generation that were suggestive of a delayed or absent preovulatory GnRH/LH surge. Indeed, the two middle treatment groups (0.1 and 1 mg/kg PCBs) exhibited relatively little fluctuation in serum LH or progesterone levels across the estrous cycle relative to control rats or those

exposed to the highest 10 mg/kg dose of A1221. The GnRH/LH surge is typically attributed to escalating levels of estradiol released by ovarian granulosa cells during the follicular phase of estrous cycle. Estradiol then stimulates GnRH release from the preoptic area via an unknown mechanism, and the GnRH surge is directly attributable for the ensuing LH surge from the anterior pituitary. Normal levels of estrogen in PCB-treated animals were apparently insufficient to elicit a normal LH surge, which implicates a central mechanism for PCB effects on LH release. In fact, there is evidence linking A1221 exposure to altered GnRH gene expression in vivo and in vitro (Gore, 2001, Gore, et al., 2002). Although in the microarray assay, the GnRH transcript did not have a p-value less than 0.01 and thus was not included in analysis, however other affected genes may play a role in the mediation of GnRH release.

In addition to altered serum LH, progesterone levels were correspondingly abnormal on the day of proestrus. Following ovulation, the ruptured follicle is transformed into a corpus luteum, which secretes progesterone. PCB-treated animals had low serum levels of progesterone on proestrus, but normal, high levels on the day of estrus. This suggests two possibilities: (1) the LH surge in PCB-exposed females was delayed, allowing for delayed ovulation and progesterone production, or (2) the LH surge is absent, but FSH (also released from the anterior pituitary prior to ovulation) induces ovulation in the absence of LH with delayed induction of corpus luteal progesterone release. Although this effect is normally attributable to LH, FSH is capable of these effects in the absence of LH (Schwartz, 1974). We did not quantify serum FSH within this dissertation research, and thus cannot make a conclusive statement on the mechanisms involved. An additional consideration is that corpus luteum tissues may also be responding abnormally to gonadotropins. These findings have strong

implications for fertility, and this is a promising route for follow-up studies on the second generation of exposed animals.

First generation (F1) exposed females did not evince altered circulating hormone levels for the hormones tested, however it is important to note that second generation (F2) females had entirely normal levels on the day of estrus, which is the only day for which F1 female serum was collected. Future studies will investigate F1 serum hormones across the estrous cycle. Additionally, F1 females showed altered sexual behaviors. Our observation of a requirement for more trials to successful mating in F1 females, described in detail in the "A1221 Effects on Paced Mating Behaviors" section of the Discussion in Research Section 2 is consistent with a dissociation of reproductive hormones and suggests that the temporal pattern of gonadotropin and sex steroid hormones may also be altered in the F1 generation. Indeed, in one mating trial not described above, an F1 female whose vaginal cytology indicated that she was already past behavioral estrus was accidentally used in a mating trial, and to my surprise, she was sexually receptive. This result suggests an uncoupling of reproductive hormones with receptive behavior.

Effects of PCBs on female paced mating behaviors

The major findings from the paced mating experiment are that PCB-exposed females are less likely to mate, given the opportunity. Treated females in the 1 mg/kg and 10 mg/kg PCB groups required almost double the number of opportunities prior to successful mating. This is even more meaningful considering that in the experimental protocol animals were only offered 4 attempts to mate, and in these two higher treatment group some females required typically 3 or 4 attempts, or never mated. This finding is

highly relevant to the continued existence and survival of wildlife species in increasingly shrinking natural habitats, for which chance mating encounters may be scarce.

Another highly significant and unexpected result of PCB exposure was the frequency of audible call emission by PCB-exposed females. Audible calls for rats are believed to convey stress and/or pain. Pain response and c-fiber nerve conduction would be excellent endpoints to address in further research. Counterintuitive to the expected "negative" effects of PCB exposure, exposed rats in the 1 mg/kg group vocalized *less* frequently than control animals. Two possible explanations for this phenomenon are (1) altered brain circuitry for the vocal production pathway, or, more likely (2) altered sensitivity to pain or novel or stressful situations. While this may appear to be an adaptive trait in the modern world, depressed stress response is highly maladaptive when considering that the required behavioral and physiological reaction to a potentially harmful situation may be compromised in exposed individuals. In future research, direct measure of circulating corticosterone levels would help to clarify these issues.

The two major findings of the sexual behaviors research may appear contradictory. The same females who were loath to mate appeared calmer when they did mate. However, it is important to recognize that the induction of mating is carefully orchestrated by the combined systemic activities of steroid hormones and central influence of neurotransmitter release, carefully timed to the female's peak in fertility. In contrast, stress response during mating, or in other situations, relies on rapid non-premeditated physiological response mediated by the corticotropin releasing hormone-adrenocorticotropic hormone-corticosterone pathway, and the hypothalamic-pituitary-adrenal (HPA) axis. Therefor, PCB disruption in these multiple

hormone systems may activate opposing signals during the induction of, and reaction to, sexual encounters.

The ability of A1221 to disrupt steroid hormone signaling likely at underlies many of the observed effects in the current body of research. Steroid hormones are key patterning agents in the developing brain, and can interact with multiple signaling pathways, neurotransmitter systems, and migratory or proliferative developmental events. Because of its known estrogenic effects, I hypothesized that Aroclor 1221 was acting in an estrogenic manner during the period of sexual differentiation of the brain. Effects on sexual behaviors, including audible vocalizations, are discussed below. Additionally, because the dopamine neurotransmitter system is a common target for PCB disruption, behavior results are interpreted with regards to possible dopaminergic disruption.

Aroclor 1221 has well-documented estrogenic effects (see General Introduction), and can also act as an anti-androgen by antagonizing activation of the androgen receptor in the presenece of dihydrotestosterone (Schrader and Cooke, 2003), or by inhibiting aromatase, the enzyme which converts testosterone into the male bioactive brain hormone estrogen (Woodhouse and Cooke, 2004). Female rats prenatally exposed to androgens exhibit decreased sexual receptivity as adults (Hoepfner and Ward, 1988), and similarly, prenatal A1221 in this dissertation research may have acted in an estrogenic manner during development with long term implications for adult sexual behaviors. The female rats exposed to A1221 prenatally in the current body of work exhibited longer mount-return latencies as adults, just as female rats treated neonatally with testosterone propionate exhibited longer event-return latencies as adults (Gans and Erskine, 2003). This could be due to an endocrine disruptive mechanism of A1221 during development.

The relationship between audible vocalizations as a measure of stress or pain suggests that audible vocalization incidence is directly related to stress response, however there is a lack of information on mating-related stress in the female rat. Audible vocalizations in the rat are associated with stress or pain response (Ardid, et al., 1993, Han, et al., 2005, Jourdan, et al., 1997, Levine, et al., 1984, Yajima, et al., 1981) and may be mediated by endogenous sex hormone fluctuations across the female estrous cycle (Kayser, et al., 1996). Thus it is possible that altered circulating hormone levels in PCB-treated rats on the day of mating might explain an altered stress response. Indeed, rats prenatally administered 17α-ethinylestradiol (Arabo, et al., 2005) or bisphenol A (Fujimoto, et al., 2006), both estrogenic chemicals, displayed increased anxiety and a depressive-like demeanor as an adult. Future research will directly assay stress response and will measure circulating corticosterone levels in female rats directly following mating.

Dopamine in the preoptic area and nucleus accumbens play a role in female display of lordosis and in the establishment of place preference and reward (Ahlenius, 1993, Becker, et al., 2001, Jenkins and Becker, 2003). The only study to investigate the effects of A1221 *in vivo* on dopaminergic endpoints found no immunohistochemical difference in the number of dopaminergic cell bodies projecting to the preoptic area (cell bodies stained with tyrosine hydroxylase antibody) (Chung, et al., 2001). No differences in lordosis were observed between groups in this dissertation research, suggesting little to no effect on tuberoinfundibular dopamine population, which is involved in female sexual behaviors. However, one unreported event of repeated spinning behavior in a single rat during a mating trial in my study has been previously documented in PCB disruption of the nucleus accumbens dopamine system (Chou, et al., 1979). It is possible that A1221

may have differentially affected dopaminergic subpopulations in experimental females, but further studies would be necessary to confirm this hypothesis because effects of PCBs on dopamine populations that are not involved in sexual behaviors would not be revealed from the endpoints measured in this study.

Effects of PCBs on gene expression in the preoptic area

The third component of this project tested global changes in gene expression across the whole genome, using preoptic area tissues. Due to the nature of the experiment (intact females, euthanized months following exposure, using very low exposure levels), subtle results were expected. I hypothesized that mainly neuroendocrine-specific genes would be altered by exposure, and that a non-linear dose response curve would again be in evidence. Given the neuroendocrine nature of the preoptic area, which is responsible for regulation of hormone production and release, there was a danger of observing overrepresentation of affected hormone-related genes merely due to their overrepresentation in the preoptic area. On the contrary, few hormone-related genes were included in the top list ($p < 0.01$). Nevertheless, disruption of even a few key genes that were members of this group have the potential to elicit large physiological effects (e.g. the estrogen receptors alpha and beta, corticotropin releasing hormone receptor, etc.)

Most of the altered genes ($p < 0.01$) identified by the microarrays belonged to cellular metabolism and intercellular communication categories, with a strong representation by cytokine and transcriptional signaling molecules. The changes in gene expression observed with this experimental protocol reflect adult gene profiles, whereas entirely different gene expression profiles are likely in the preoptic area if assayed closer to the

timing of exposure. Because of the known hormone mimic effects of A1221, it is probable that the immediate response to PCB exposure is strongly hormone related, causing altered development and brain patterning of hormone signaling pathways that emerge as altered situational responsiveness in the adult. However, PCBs can work through many other avenues, and gene expression in cellular physiology pathways remains affected in the adult. Preoptic areas of P1 culled pups, both male and female, were collected and further microarray research would be highly beneficial towards further exploration of the relationship between early developmental gene expression alteration and later adult expression following compensation and maturation.

Of the hormone-related genes with $p < 0.01$, three general groups emerged: estrogen-related genes, thyroid hormone-related genes, and corticotropin-releasing hormone-related genes. All of these hormone systems have been documented as affected by PCB exposure in previous research (see the Introduction to this dissertation). In the context of the findings of this dissertation research, the observed increase in estrogen receptor expression may signify altered circulating estrogen levels, perhaps on the day of proestrus—1 day prior to euthanasia, or an altered response to serum estrogen. Serum estrogen on the day of euthanasia was normal, as discussed previously, however the transcriptionally-mediated effects of estradiol's downstream activity may take hours to days to present, which allows for possible altered estradiol levels on other days of the estrous cycle.

Altered thyroid hormone receptor-associated protein, and thyrotropin releasing hormone receptor 2 expression was observed for the 1 mg/kg and 10 mg/kg treament groups, respectively. Whereas in the adult, thyroid hormones regulate metabolism, in the fetus they serve an essential role in guiding brain development (Anderson, et al., 2003). Because expression of

these thyroid hormone-related genes was altered in the adult, it is possible that prenatal thyroid hormone signaling was also affected. Further research on prenatal PCBs is needed to measure adult serum thyroid hormone levels, and to investigate neonatal brain morphology for signs of hypo- or hyperthyroidism.

The downregulation of corticosterone releasing hormone-related genes in the 1 mg/kg group is highly relevant considering that this same treated group exhibited decreased audible vocalizations, an indicator of the stress response during mating trials. Because tissues were collected the day following mating, the two findings are temporally related, and may represent a cause-and-effect scenario. Future research would do well to investigate stress response, gene expression, and circulating stress hormone levels for animals in both mating and non-mating situations to determine if the observed altered stress response is specific to mating situations.

Results from TRE analysis show that the most common TREs to the top 50 genes are either ubiquitous across the genome, or else are blood and brain development-related. This finding mirrors the observed depressed expression of blood- and immune-related genes, and of vascular smooth muscle-related genes that may signify lower than normal vascularization of the preoptic area of the brain in PCB-exposed females. Upregulation of several genes related to blood-brain barrier integrity could be related. One explanation may be that prenatal intoxication caused a reactive strengthening of the blood-brain barrier in PCB-exposed animals, and that either direct PCB exposure acting at blood-related transcriptional response elements or indirect effects of signaling by blood-brain barrier tissues possibly altered growth of blood vessels in this region. Decreased vascularization of this region might also have indirect effects on sexual

behaviors, if increased metabolic needs of this brain nucleus during sexual stimulation are not fulfilled by the delivery of blood and nutrients.

A number of genes characterized as disease risk factors were detected amongst the most significantly affected genes in the microarray experiment results. Clearly, follow-up quantitative real time PCR is required to clarify the actual differences in gene expression between treatment groups, however the power to generate testable hypotheses from genome-wide expression patterns is an invaluable tool for guiding future endeavors in the study of female sexuality and beyond. The results of this microarray experiment should thus be taken in that context, and a creative outlook towards future problem solving of trends in human health and wildlife survival, in the context of environmental toxicology.

Non-linear dose-response curves and low-dose exposures

Non-linear dose-response curves were common to all three research areas included in this dissertation: development & fecundity, sexual behaviors, and gene expression. A hallmark of steroid hormone-like endocrine disrupting chemicals, this type of dose-response relationship renders it difficult to assign minimum toxic dose levels and to predict medical outcomes of toxicant exposure. In paced mating assays, a commonly observed pattern was for the lowest or median treatment to elicit effects in one direction and the 10 mg/kg group to elicit effects in the opposite direction, resulting in significant differences amongst treatment groups, but not with the control group. Differences amongst treated groups were mostly omitted from the results of each research section but such differences may still be biologically relevant

Dose-response curve linearity tests represent a considerable statistical challenge. For the developmental and behavioral results, the data

sets were not large enough to conduct direct assays of the presence of U-shaped or inverted U-shaped dose-response curves, which would signifify a hormetic or steroid-hormone like mechanism. Instead, the conservative term "non-linear" is employed based on the observed high incidence of strong treatment effects at the middle treatment levels across multiple endpoints. However, the large number of data points generated by whole genome microarray renders it possible to submit a large data set of dose response curves to a cluster analysis program in order to find patterns common to the largest groupings of genes. The results of this analysis show that the most common dose-response curves are U-shaped, and thus that 1221 has a steroid hormone-like mechanism of action on adult gene expression.

Implication of experimental data for "natural" populations

The overall picture of this dissertation research is to present a global biological snapshot of the effects of prenatal PCB exposure on the sexual "wellness" of adult female animals. These females appear normal when judging their size and development, however clear abnormalities emerge when their physiology and gene expression are closely scrutinized, and when they are placed into situations that test their reactive behaviors, such as the opportunity to mate. This experimental protocol was conservative in that female pups with the median anogenital distances (a measure of masculinization following EDC exposure) were selected as experimental animals. This methodology disincluded females with stronger indications of masculinization as might be observed following exposure to an estrogenic chemical.

Low levels of toxicants like those used in this dissertation research are likely to affect certain individuals more strongly than others. Over the spread of a large population, such as with humans, PCB exposure will most likely

reshape the normal curve instead of creating distinct peaks of an abnormal phenotype at the distal edges of the curve. Thus, the peak will become flatter and broader as the incidence of previously rare traits is increased with a conjoined decrease in the number of average individuals. Selecting experimental animals known to be grouped close to the mean thus represents a conservative approach to investigating the question of low-level PCB exposure. Despite these limitations, I observed significant differences between treated groups vs. control, in endpoints highly relevant to female sexuality.

Overall Conclusion

In this series of experiments, female rats were exposed to ecologically relevant low levels of A1221 during the period of brain sexual differentiation. The exposed generation appeared normal in physiology and reproductive maturation, although they exhibited reluctance to mate, and decreased stress response to mating situations. Gene expression profiles of the POA revealed that even two months after prenatal exposure to A1221, a number of genes related to immune, blood, and hormone functions were altered. Some of these changes may underlie the differences in physiology and behavior seen in the F1 generation. The highest percentage of altered genes were downregulated in the 0.1 and 1 mg/kg groups compared to control rats, whereas in the 10 mg/kg group, more genes were upregulated compared to control animals. This finding suggests non-traditional dose-response curves and is consistent with the non-linearity of effects of A1221 on reproductive physiology and behavior. Altered hormone-related gene expression in the F1 may be implicated in observed depressed hormone levels of the F2 females if an inappropriate hormonal environment was present in the pregnant dam during fetal and embryonic development of the F2, or if F1 dams had abnormal maternal behaviors towards their F2 offspring. In future studies, our laboratory will investigate effects of prenatal PCBs on reproductive hormones across the estrous cycles of at least three generations of rats, to further understand transgenerational effects, and we will also determine if alterations in maternal behavior may occur that may impart epigenetic modifications to the offspring, as is well-established for manipulations of the HPA axis.

Current trends in toxicological research focus on PCBs that remain in measurable quantities in human tissues and the environment, thereby underestimating the impact of toxicants such as A1221. It is not impossible to

detect the fingerprint of exposure for A1221 or other endocrine disrupting chemicals even after the toxicant is no longer present in measurable quantities. The field of toxicogenomics permits the compilation of gene profile libraries documenting global effects of toxicant exposure on gene expression within an organism. This work is being pursued currently for PCB effects on amphibians (Jelaso, et al., 2002, Jelaso, et al., 2003), but application to mammals is rare. With this current dissertation research, I provide a bank of information including developmental, behavioral, and toxicogenomic endpoints to characterize prenatal exposure to one PCB mixture at one developmental time point. The later addition of many more studies of this format of toxicological research will greatly benefit both science and medicine, as patterns begin to emerge linking toxin exposures and disease trends or susceptibilities.

Bibliography

(Atsdr), A.F.T.S.A.D.R. (2000) Toxicological profile for polychlorinated biphenyls (PCBs). Journal

Agullo, L., Camara, B., Martinez, P., Latorre, V. and Seeger, M. (2007) Response to (chloro)biphenyls of the polychlorobiphenyl-degrader Burkholderia xenovorans LB400 involves stress proteins also induced by heat shock and oxidative stress. FEMS Microbiol Lett 267, 167-75.

Ahlenius, S. (1993) Brain monoaminergic neurotransmission in the mediation of lordosis behavior in the female rat. Neurosci Biobehav Rev 17, 43-9.

Ahmad, S.U., Tariq, S., Jalali, S. and Ahmad, M.M. (2003) Environmental pollutant Aroclor 1242 (PCB) disrupts reproduction in adult male rhesus monkeys (Macaca mulatta). Environ Res 93, 272-8.

Aldenhoff, J.B., Gruol, D.L., Rivier, J., Vale, W. and Siggins, G.R. (1983) Corticotropin releasing factor decreases postburst hyperpolarizations and excites hippocampal neurons. Science 221, 875-7.

Aluru, N., Jorgensen, E.H., Maule, A.G. and Vijayan, M.M. (2004) PCB disruption of the hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor downregulation in anadromous Arctic charr. Am J Physiol Regul Integr Comp Physiol 287, R787-93.

Aluru, N. and Vijayan, M.M. (2006) Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. Endocrinology 147, 1895-903.

Anderson, G.W., Schoonover, C.M. and Jones, S.A. (2003) Control of thyroid hormone action in the developing rat brain. Thyroid 13, 1039-56.

Angus, W.G. and Contreras, M.L. (1996) Effects of polychlorinated biphenyls on dopamine release from PC12 cells. Toxicol Lett 89, 191-9.

Anway, M.D., Cupp, A.S., Uzumcu, M. and Skinner, M.K. (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466-9.

Anway, M.D., Leathers, C. and Skinner, M.K. (2006) Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology 147, 5515-23.

Aoki, Y. (2001) Polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans as endocrine disrupters--what we have learned from Yusho disease. Environ Res 86, 2-11.

Arabo, A., Lefebvre, M., Fermanel, M. and Caston, J. (2005) Administration of 17alpha-ethinylestradiol during pregnancy elicits modifications of maternal behavior

and emotional alteration of the offspring in the rat. Brain Res Dev Brain Res 156, 93-103.

Arcaro, K.F., Yi, L., Seegal, R.F., Vakharia, D.D., Yang, Y., Spink, D.C., Brosch, K. and Gierthy, J.F. (1999) 2,2',6,6'-Tetrachlorobiphenyl is estrogenic in vitro and in vivo. J Cell Biochem 72, 94-102.

Ardid, D., Jourdan, D., Eschalier, A., Arabia, C. and Le Bars, D. (1993) Vocalization elicited by activation of A delta- and C-fibres in the rat. Neuroreport 5, 105-8.

Arena, S.M., Greeley, E.H., Halbrook, R.S., Hansen, L.G. and Segre, M. (2003) Biological effects of gestational and lactational PCB exposure in neonatal and juvenile C57BL/6 mice. Arch Environ Contam Toxicol 44, 272-80.

Arnold, D.L., Bryce, F., Karpinski, K., Mes, J., Fernie, S., Tryphonas, H., Truelove, J., Mcguire, P.F., Burns, D., Tanner, J.R. and Et Al. (1993) Toxicological consequences of Aroclor 1254 ingestion by female rhesus (Macaca mulatta) monkeys. Part 1B. Prebreeding phase: clinical and analytical laboratory findings. Food Chem Toxicol 31, 811-24.

Arnold, D.L., Bryce, F., Mcguire, P.F., Stapley, R., Tanner, J.R., Wrenshall, E., Mes, J., Fernie, S., Tryphonas, H., Hayward, S. and Et Al. (1995) Toxicological consequences of aroclor 1254 ingestion by female rhesus (Macaca mulatta) monkeys. Part 2. Reproduction and infant findings. Food Chem Toxicol 33, 457-74.

Aubert, M.L., Begeot, M., Winiger, B.P., Morel, G., Sizonenko, P.C. and Dubois, P.M. (1985) Ontogeny of hypothalamic luteinizing hormone-releasing hormone (GnRH) and pituitary GnRH receptors in fetal and neonatal rats. Endocrinology 116, 1565-76.

Aubrecht, J., Rugo, R. and Schiestl, R.H. (1995) Carcinogens induce intrachromosomal recombination in human cells. Carcinogenesis 16, 2841-6.

Bae, J., Mousa, M.A., Quensen, J.F., 3rd, Boyd, S.A. and Loch-Caruso, R. (2001) Stimulation of contraction of pregnant rat uterus in vitro by non-dechlorinated and microbially dechlorinated mixtures of polychlorinated biphenyls. Environ Health Perspect 109, 275-82.

Bansal, R., You, S.H., Herzig, C.T. and Zoeller, R.T. (2005) Maternal thyroid hormone increases HES expression in the fetal rat brain: an effect mimicked by exposure to a mixture of polychlorinated biphenyls (PCBs). Brain Res Dev Brain Res 156, 13-22.

Barfield, M.A. and Lisk, R.D. (1970) Advancement of behavioral estrus by subcutaneous injection of progesterone in the 4-day cyclic rat. Endocrinology 87, 1096-8.

Barton, H.A. and Andersen, M.E. (1998) A model for pharmacokinetics and physiological feedback among hormones of the testicular-pituitary axis in adult male rats: a framework for evaluating effects of endocrine active compounds. Toxicol Sci 45, 174-87.

Basha, M.R., Braddy, N.S., Zawia, N.H. and Kodavanti, P.R. (2006) Ontogenetic alterations in prototypical transcription factors in the rat cerebellum and hippocampus following perinatal exposure to a commercial PCB mixture. Neurotoxicology 27, 118-24.

Bastomsky, C.H., Murthy, P.V. and Banovac, K. (1976) Alterations in thyroxine metabolism produced by cutaneous application of microscope immersion oil: effects due to polychlorinated biphenyls. Endocrinology 98, 1309-14.

Battershill, J.M. (1994) Review of the safety assessment of polychlorinated biphenyls (PCBs) with particular reference to reproductive toxicity. Hum Exp Toxicol 13, 581-97.

Becker, J.B., Rudick, C.N. and Jenkins, W.J. (2001) The role of dopamine in the nucleus accumbens and striatum during sexual behavior in the female rat. J Neurosci 21, 3236-41.

Becu-Villalobos, D., Gonzalez Iglesias, A., Diaz-Torga, G., Hockl, P. and Libertun, C. (1997) Brain sexual differentiation and gonadotropins secretion in the rat. Cell Mol Neurobiol 17, 699-715.

Behan, D.P., Heinrichs, S.C., Troncoso, J.C., Liu, X.J., Kawas, C.H., Ling, N. and De Souza, E.B. (1995) Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature 378, 284-7.

Bemis, J.C. and Seegal, R.F. (2000) Polychlorinated biphenyls and methylmercury alter intracellular calcium concentrations in rat cerebellar granule cells. Neurotoxicology 21, 1123-34.

Bemis, J.C. and Seegal, R.F. (2004) PCB-induced inhibition of the vesicular monoamine transporter predicts reductions in synaptosomal dopamine content. Toxicol Sci 80, 288-95.

Boersma, E.R. and Lanting, C.I. (2000) Environmental exposure to polychlorinated biphenyls (PCBs) and dioxins. Consequences for longterm neurological and cognitive development of the child lactation. Adv Exp Med Biol 478, 271-87.

Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H.M., Jiang, J., Marinelli, B., Pesatori, A.C., Bertazzi, P.A. and Yang, A.S. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 67, 876-80.

Bolstad, B.M., Irizarry, R.A., Astrand, M. and Speed, T.P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19, 185-93.

Borszcz, G.S. (1995) Pavlovian conditional vocalizations of the rat: a model system for analyzing the fear of pain. Behav Neurosci 109, 648-62.

Bowers, W.J., Nakai, J.S., Chu, I., Wade, M.G., Moir, D., Yagminas, A., Gill, S., Pulido, O. and Meuller, R. (2004) Early developmental neurotoxicity of a PCB/organoclorine mixture in rodents after gestational and lactational exposure. Toxicol Sci 77, 51-62.

Bowman, R.E., Heironimus, M.P. and Barsotti, D.A. (1981) Locomotor hyperactivity in PCB-exposed rhesus monkeys. Neurotoxicology 2, 251-68.

Breedlove, S.M. (1992) Sexual dimorphism in the vertebrate nervous system. J Neurosci 12, 4133-42.

Brevini, T.A., Zanetto, S.B. and Cillo, F. (2005) Effects of endocrine disruptors on developmental and reproductive functions. Curr Drug Targets Immune Endocr Metabol Disord 5, 1-10.

Brezner, E., Terkel, J. and Perry, A.S. (1984) The effect of Aroclor 1254 (PCB) on the physiology of reproduction in the female rat--I. Comp Biochem Physiol C 77, 65-70.

Brouwer, A. and Van Den Berg, K.J. (1986) Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxin. Toxicol Appl Pharmacol 85, 301-12.

Brouwer, A. (1989) Inhibition of thyroid hormone transport in plasma of rats by polychlorinated biphenyls. Arch Toxicol Suppl 13, 440-5.

Brouwer, A., Longnecker, M.P., Birnbaum, L.S., Cogliano, J., Kostyniak, P., Moore, J., Schantz, S. and Winneke, G. (1999) Characterization of potential endocrine-related health effects at low-dose levels of exposure to PCBs. Environ Health Perspect 107 Suppl 4, 639-49.

Brunnberg, S., Pettersson, K., Rydin, E., Matthews, J., Hanberg, A. and Pongratz, I. (2003) The basic helix-loop-helix-PAS protein ARNT functions as a potent coactivator of estrogen receptor-dependent transcription. Proc Natl Acad Sci U S A 100, 6517-22.

Burse, V.W., Patterson, D.G., Jr., Brock, J.W. and Needham, L.L. (1996) Selected analytical methods used at the Centers for Disease Control and Prevention for measuring environmental pollutants in serum. Toxicol Ind Health 12, 481-98.

Buterin, T., Koch, C. and Naegeli, H. (2006) Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis

Butterworth, F.M., Pandey, P., Mcgowen, R.M., Ali-Sadat, S. and Walia, S. (1995) Genotoxicity of polychlorinated biphenyls (PCBs): recombinogenesis by biotransformation products. Mutat Res 342, 61-9.

Byrne, J.J. and Sepkovic, D.W. (1987) Inhibition of monovalent cation transport across the cell membrane by polychlorinated biphenyl but not by polybrominated biphenyl. Arch Environ Contam Toxicol 16, 573-7.

Calabrese, E.J. and Baldwin, L.A. (2001a) U-shaped dose-responses in biology, toxicology, and public health. Annu Rev Public Health 22, 15-33.

Calabrese, E.J. and Baldwin, L.A. (2001b) Hormesis: U-shaped dose responses and their centrality in toxicology. Trends Pharmacol Sci 22, 285-91.

Calabrese, E.J. and Baldwin, L.A. (2003) Toxicology rethings its central belief. Nature 421, 691-2.

Calvino, B., Besson, J.M., Boehrer, A. and Depaulis, A. (1996) Ultrasonic vocalization (22-28 kHz) in a model of chronic pain, the arthritic rat: effects of analgesic drugs. Neuroreport 7, 581-4.

Carlson, D.B. and Williams, D.E. (2001) 4-hydroxy-2',4',6'-trichlorobiphenyl and 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl are estrogenic in rainbow trout. Environ Toxicol Chem 20, 351-8.

Carpenter, D.O. (1998) Polychlorinated biphenyls and human health. Int J Occup Med Environ Health 11, 291-303.

Carpenter, D.O., Decaprio, A.P., O'hehir, D., Akhtar, F., Johnson, G., Scrudato, R.J., Apatiki, L., Kava, J., Gologergen, J., Miller, P.K. and Eckstein, L. (2005) Polychlorinated biphenyls in serum of the Siberian Yupik people from St. Lawrence Island, Alaska. Int J Circumpolar Health 64, 322-35.

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. and Werner, T. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933-42.

Cassell, D.S. (Year) A Randomized-test Wrapper for SAS PROCs. SAS SUGI27,Orlando, FL

Caudle, W.M., Richardson, J.R., Delea, K.C., Guillot, T.S., Wang, M., Pennell, K.D. and Miller, G.W. (2006) Polychlorinated Biphenyl (PCB)-Induced Reduction of Dopamine Transporter Expression as a Precursor to Parkinson's Disease-associated Dopamine Toxicity. Toxicol Sci

Champagne, F. and Meaney, M.J. (2001) Like mother, like daughter: evidence for non-genomic transmission of parental behavior and stress responsivity. Prog Brain Res 133, 287-302.

Champagne, F.A., Weaver, I.C., Diorio, J., Dymov, S., Szyf, M. and Meaney, M.J. (2006) Maternal care associated with methylation of the estrogen receptor-alpha1b promoter and estrogen receptor-alpha expression in the medial preoptic area of female offspring. Endocrinology 147, 2909-15.

Chang, H.S., Anway, M.D., Rekow, S.S. and Skinner, M.K. (2006) Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. Endocrinology 147, 5524-41.

Chauhan, K.R., Kodavanti, P.R. and Mckinney, J.D. (2000) Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol Appl Pharmacol 162, 10-21.

Chen, R.C., Tang, S.Y., Miyata, H., Kashimoto, T., Chang, Y.C., Chang, K.J. and Tung, T.C. (1985) Polychlorinated biphenyl poisoning: correlation of sensory and motor nerve conduction, neurologic symptoms, and blood levels of polychlorinated biphenyls, quaterphenyls, and dibenzofurans. Environ Res 37, 340-8.

Chen, Y.C., Guo, Y.L. and Hsu, C.C. (1992) Cognitive development of children prenatally exposed to polychlorinated biphenyls (Yu-Cheng children) and their siblings. J Formos Med Assoc 91, 704-7.

Choksi, N.Y., Kodavanti, P.R., Tilson, H.A. and Booth, R.G. (1997) Effects of polychlorinated biphenyls (PCBs) on brain tyrosine hydroxylase activity and dopamine synthesis in rats. Fundam Appl Toxicol 39, 76-80.

Chou, S.M., Miike, T., Payne, W.M. and Davis, G.J. (1979) Neuropathology of "spinning syndrome" induced by prenatal intoxication with a PCB in mice. Ann N Y Acad Sci 320, 373-95.

Choudhury, R.C., Jagdale, M.B. and Misra, S. (2000) Potential transmission of the cytogenetic effects of cisplatin in the male germline cells of Swiss mice. J Chemother 12, 352-9.

Chu, I., Villeneuve, D.C., Yagminas, A., Lecavalier, P., Poon, R., Feeley, M., Kennedy, S.W., Seegal, R.F., Hakansson, H., Ahlborg, U.G. and Et Al. (1994) Subchronic toxicity of 3,3',4,4',5-pentachlorobiphenyl in the rat. I. Clinical, biochemical, hematological, and histopathological changes. Fundam Appl Toxicol 22, 457-68.

Chu, I., Villeneuve, D.C., Yagminas, A., Lecavalier, P., Hakansson, H., Ahlborg, U.G., Valli, V.E., Kennedy, S.W., Bergman, A., Seegal, R.F. and Et Al. (1995) Toxicity of PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 118 (2,3',4,4'5-pentachlorobiphenyl) in the rat following subchronic dietary exposure. Fundam Appl Toxicol 26, 282-92.

Chu, I., Villeneuve, D.C., Yagminas, A., Lecavalier, P., Poon, R., Feeley, M., Kennedy, S.W., Seegal, R.F., Hakansson, H., Ahlborg, U.G., Valli, V.E. and Bergman, A. (1996) Toxicity of 2,2',4,4',5,5'-hexachlorobiphenyl in rats: effects following 90-day oral exposure. J Appl Toxicol 16, 121-8.

Chu, I., Poon, R., Yagminas, A., Lecavalier, P., Hakansson, H., Valli, V.E., Kennedy, S.W., Bergman, A., Seegal, R.F. and Feeley, M. (1998) Subchronic toxicity of PCB 105 (2,3,3',4,4'-pentachlorobiphenyl) in rats. J Appl Toxicol 18, 285-92.

Chung, D. and Loch Caruso, R. (2005) 2,2'-Dichlorobiphenyl decreases amplitude and synchronization of uterine contractions through MAPK1-mediated phosphorylation of GJA1 (connexin43) and inhibition of myometrial gap junctions. Biol Reprod 73, 974-82.

Chung, Y.W. and Clemens, L.G. (1999) Effects of perinatal exposure to polychlorinated biphenyls on development of female sexual behavior. Bull Environ Contam Toxicol 62, 664-70.

Chung, Y.W., Nunez, A.A. and Clemens, L.G. (2001) Effects of neonatal polychlorinated biphenyl exposure on female sexual behavior. Physiol Behav 74, 363-70.

Coburn, C.G., Gillard, E.R. and Curras-Collazo, M.C. (2005) Dietary exposure to aroclor 1254 alters central and peripheral vasopressin release in response to dehydration in the rat. Toxicol Sci 84, 149-56.

Colciago, A., Celotti, F., Pravettoni, A., Mornati, O., Martini, L. and Negri-Cesi, P. (2005) Dimorphic expression of testosterone metabolizing enzymes in the hypothalamic area of developing rats. Brain Res Dev Brain Res 155, 107-16.

Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A. and Balaguer, P. (1997) Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. Toxicol Appl Pharmacol 145, 111-23.

Cooper, R.L. and Linnoila, M. (1977) Sexual behavior in aged, noncycling female rats. Physiol Behav 18, 573-6.

Coopersmith, C. and Erskine, M.S. (1994) Influence of paced mating and number of intromissions on fertility in the laboratory rat. J Reprod Fertil 102, 451-8.

Cope, R.B., Imsilp, K., Morrow, C.K., Hartman, J., Schaeffer, D.J. and Hansen, L.G. (2003) Exposure to soil contaminated with an environmental PCB/PCDD/PCDF mixture modulates ultraviolet radiation-induced non-melanoma skin carcinogenesis in the Crl:SKH1-hrBR hairless mouse. Cancer Lett 191, 145-54.

Corrigan, F.M., Murray, L., Wyatt, C.L. and Shore, R.F. (1998) Diorthosubstituted polychlorinated biphenyls in caudate nucleus in Parkinson's disease. Exp Neurol 150, 339-42.

Corrigan, F.M., Wienburg, C.L., Shore, R.F., Daniel, S.E. and Mann, D. (2000) Organochlorine insecticides in substantia nigra in Parkinson's disease. J Toxicol Environ Health A 59, 229-34.

Crews, D., Bergeron, J.M. and Mclachlan, J.A. (1995) The role of estrogen in turtle sex determination and the effect of PCBs. Environ Health Perspect 103 Suppl 7, 73-7.

Crews, D., Willingham, E. and Skipper, J.K. (2000) Endocrine disruptors: present issues, future directions. Q Rev Biol 75, 243-60.

Crews, D., Lou, W., Fleming, A. and Ogawa, S. (2006) From gene networks underlying sex determination and gonadal differentiation to the development of neural networks regulating sociosexual behavior. Brain Res

Crofton, K.M., Kodavanti, P.R., Derr-Yellin, E.C., Casey, A.C. and Kehn, L.S. (2000) PCBs, thyroid hormones, and ototoxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. Toxicol Sci 57, 131-40.

Csaba, G., Karabelyos, C., Inczefi-Gonda, A. and Pallinger, E. (2005) Three-generation investigation on serotonin content in rat immune cells long after beta-endorphin exposure in late pregnancy. Horm Metab Res 37, 172-7.

Cummings, J.A., Nunez, A.A. and Clemens, L.G. (2005) A cross-fostering analysis of the effects of PCB 77 on the maternal behavior of rats. Physiol Behav 85, 83-91.

Cummings, S., Elde, R., Ells, J. and Lindall, A. (1983) Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: an immunohistochemical study. J Neurosci 3, 1355-68.

Daftary, S.S. and Gore, A.C. (2003) Developmental changes in hypothalamic insulin-like growth factor-1: relationship to gonadotropin-releasing hormone neurons. Endocrinology 144, 2034-45.

Dai, Y.S., Cserjesi, P., Markham, B.E. and Molkentin, J.D. (2002) The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism. J Biol Chem 277, 24390-8.

De Kloet, E.R., Vreugdenhil, E., Oitzl, M.S. and Joels, M. (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev 19, 269-301.

De Krey, G.K., Baecher-Steppan, L., Deyo, J.A., Smith, B. and Kerkvliet, N.I. (1993) Polychlorinated biphenyl-induced immune suppression: castration, but not adrenalectomy or RU 38486 treatment, partially restores the suppressed cytotoxic T lymphocyte response to alloantigen. J Pharmacol Exp Ther 267, 308-15.

De Krey, G.K., Hollingshead, N.C., Kerkvliet, N.I. and Smith, B.B. (1994) Suppression of prolactin and cytotoxic T-lymphocyte activity in PCB-treated mice. Int J Immunopharmacol 16, 251-7.

De Smet, F., Mathys, J., Marchal, K., Thijs, G., De Moor, B. and Moreau, Y. (2002) Adaptive quality-based clustering of gene expression profiles. Bioinformatics 18, 735-46.

Decastro, B.R., Korrick, S.A., Spengler, J.D. and Soto, A.M. (2006) Estrogenic activity of polychlorinated biphenyls present in human tissue and the environment. Environ Sci Technol 40, 2819-25.

Del Rio Gomez, I., Marshall, T., Tsai, P., Shao, Y.S. and Guo, Y.L. (2002) Number of boys born to men exposed to polychlorinated byphenyls. Lancet 360, 143-4.

Denham, M., Schell, L.M., Deane, G., Gallo, M.V., Ravenscroft, J. and Decaprio, A.P. (2005) Relationship of lead, mercury, mirex, dichlorodiphenyldichloroethylene, hexachlorobenzene, and polychlorinated biphenyls to timing of menarche among Akwesasne Mohawk girls. Pediatrics 115, e127-34.

Desaulniers, D., Leingartner, K., Wade, M., Fintelman, E., Yagminas, A. and Foster, W.G. (1999) Effects of acute exposure to PCBs 126 and 153 on anterior pituitary and thyroid hormones and FSH isoforms in adult Sprague Dawley male rats. Toxicol Sci 47, 158-69.

Dewitt, J.C., Millsap, D.S., Yeager, R.L., Heise, S.S., Sparks, D.W. and Henshel, D.S. (2006) External heart deformities in passerine birds exposed to environmental mixtures of polychlorinated biphenyls during development. Environ Toxicol Chem 25, 541-51.

Dhandapani, K.M. and Brann, D.W. (2002) Estrogen-astrocyte interactions: implications for neuroprotection. BMC Neurosci 3, 6.

Dohler, K.D. (1991) The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus. Int Rev Cytol 131, 1-57.

Donahue, D.A., Dougherty, E.J. and Meserve, L.A. (2004) Influence of a combination of two tetrachlorobiphenyl congeners (PCB 47; PCB 77) on thyroid status, choline acetyltransferase (ChAT) activity, and short- and long-term memory in 30-day-old Sprague-Dawley rats. Toxicology 203, 99-107.

Drenth, H.J., Bouwman, C.A., Seinen, W. and Van Den Berg, M. (1998) Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. Toxicol Appl Pharmacol 148, 50-5.

Durham, S.K. and Brouwer, A. (1990) 3,4,3',4'-Tetrachlorobiphenyl distribution and induced effects in the rat adrenal gland. Localization in the zona fasciculata. Lab Invest 62, 232-9.

Edmonds, S., Zoloth, S.R. and Adler, N.T. (1972) Storage of copulatory stimulation in the female rat. Physiol Behav 8, 161-4.

Elkin, B.T. and Bethke, R.W. (1995) Environmental contaminants in caribou in the Northwest Territories, Canada. Sci Total Environ 160-161, 307-21.

Endo, F., Monsees, T.K., Akaza, H., Schill, W.B. and Pflieger-Bruss, S. (2003) Effects of single non-ortho, mono-ortho, and di-ortho chlorinated biphenyls on cell functions and proliferation of the human prostatic carcinoma cell line, LNCaP. Reprod Toxicol 17, 229-36.

Eriksson, P., Lundkvist, U. and Fredriksson, A. (1991) Neonatal exposure to 3,3',4,4'-tetrachlorobiphenyl: changes in spontaneous behaviour and cholinergic muscarinic receptors in the adult mouse. Toxicology 69, 27-34.

Erskine, M.S. (1985) Effects of paced coital stimulation on estrus duration in intact cycling rats and ovariectomized and ovariectomized-adrenalectomized hormone-primed rats. Behav Neurosci 99, 151-61.

Erskine, M.S. (1992) Pelvic and pudendal nerves influence the display of paced mating behavior in response to estrogen and progesterone in the female rat. Behav Neurosci 106, 690-7.

Erskine, M.S., Lehmann, M.L., Cameron, N.M. and Polston, E.K. (2004) Co-regulation of female sexual behavior and pregnancy induction: an exploratory synthesis. Behav Brain Res 153, 295-315.

Esper, R.M., Pankonin, M.S. and Loeb, J.A. (2006) Neuregulins: versatile growth and differentiation factors in nervous system development and human disease. Brain Res Brain Res Rev 51, 161-75.

Eum, S.Y., Rha, G.B., Hennig, B. and Toborek, M. (2006) c-Src Is the Primary Signaling Mediator of Polychlorinated Biphenyl-Induced Interleukin-8 Expression in a Human Microvascular Endothelial Cell Line. Toxicol Sci 92, 311-20.

Fernie, K., Bortolotti, G., Drouillard, K., Smits, J. and Marchant, T. (2003) Developmental toxicity of in ovo exposure to polychlorinated biphenyls: II. Effects of maternal or paternal exposure on second-generation nestling american kestrels. Environ Toxicol Chem 22, 2688-94.

Fernie, K.J., Smits, J.E., Bortolotti, G.R. and Bird, D.M. (2001) In ovo exposure to polychlorinated biphenyls: reproductive effects on second-generation American kestrels. Arch Environ Contam Toxicol 40, 544-50.

Fielden, M.R., Chen, I., Chittim, B., Safe, S.H. and Zacharewski, T.R. (1997) Examination of the estrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2',6'-pentachloro-4-biphenylol (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). Environ Health Perspect 105, 1238-48.

Fisher, S.A., Bortolotti, G.R., Fernie, K.J., Bird, D.M. and Smits, J.E. (2005) Behavioral variation and its consequences during incubation for American kestrels exposed to polychlorinated biphenyls. Ecotoxicol Environ Saf

Flouriot, G., Pakdel, F., Ducouret, B. and Valotaire, Y. (1995) Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. J Mol Endocrinol 15, 143-51.

Frame, G. (1997a) A collaborative study of 209 PCB congeners and 6 Aroclors on 20 diffrent HRGC columns, 2. Semi-quantitative Aroclor congener distributions. Fresenius J Anal Chem 357, 714-22.

Frame, G.M. (1996) Complete PCB Congener Distributions for 17 Aroclor Mixtures Determined by 3 HRGC Systems Optimized for Comprehensive, Quantitative, Congener-Specific Analysis. J. HIgh Resol. Chromatogr. 19,

Frame, G.M. (1997b) A collaborative study of 209 PCB congeners and 6 Aroclors on 20 different HRGC columns. Fresenius Journal of Analytical Chemistry 357, 714-22.

Fujimoto, T., Kubo, K. and Aou, S. (2006) Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res 1068, 49-55.

Fujiwara, M. and Kuriyama, K. (1977) Effect of PCB (polychlorobiphenyls) on on L-ascorbic acid, pyridoxal phosphate and riboflavin contents in various organs and on hepatic metabolism of L-ascorbic acid in the rat. Jpn J Pharmacol 27, 621-7.

Furst, P. (2006) Dioxins, polychlorinated biphenyls and other organohalogen compounds in human milk. Levels, correlations, trends and exposure through breastfeeding. Mol Nutr Food Res 50, 922-33.

Gafni, J., Wong, P.W. and Pessah, I.N. (2004) Non-coplanar 2,2',3,5',6-pentachlorobiphenyl (PCB 95) amplifies ionotropic glutamate receptor signaling in embryonic cerebellar granule neurons by a mechanism involving ryanodine receptors. Toxicol Sci 77, 72-82.

Gallavan, R.H., Jr., Holson, J.F., Stump, D.G., Knapp, J.F. and Reynolds, V.L. (1999) Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 13, 383-90.

Gans, S. and Erskine, M.S. (2003) Effects of neonatal testosterone treatment on pacing behaviors and development of a conditioned place preference. Horm Behav 44, 354-64.

Gauger, K.J., Kato, Y., Haraguchi, K., Lehmler, H.J., Robertson, L.W., Bansal, R. and Zoeller, R.T. (2004) Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. Environ Health Perspect 112, 516-23.

Gellert, R.J. (1978) Uterotrophic activity of polychlorinated biphenyls (PCB) and induction of precocious reproductive aging in neonatally treated female rats. Environ Res 16, 123-30.

Gillette, D.M., Corey, R.D., Lowenstine, L.J. and Shull, L.R. (1987) Comparative toxicology of tetrachlorobiphenyls in mink and rats. II. Pathology. Fundam Appl Toxicol 8, 15-22.

Goldey, E.S., Kehn, L.S., Lau, C., Rehnberg, G.L. and Crofton, K.M. (1995) Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. Toxicol Appl Pharmacol 135, 77-88.

Goldman, D. and Yawetz, A. (1991) Cytochrome P-450 mediated metabolism of progesterone by adrenal microsomes of PCB-treated and untreated barn owl (Tyto alba) and marsh turtle (Mauremys caspica) in comparison with the guinea-pig. Comp Biochem Physiol C 99, 251-5.

Gore, A.C. and Roberts, J.L. (1994) Regulation of gonadotropin-releasing hormone gene expression by the excitatory amino acids kainic acid and N-methyl-D,L-aspartate in the male rat. Endocrinology 134, 2026-31.

Gore, A.C., Roberts, J.L. and Gibson, M.J. (1999) Mechanisms for the regulation of gonadotropin-releasing hormone gene expression in the developing mouse. Endocrinology 140, 2280-7.

Gore, A.C. (2001) Environmental toxicant effects on neuroendocrine function. Endocrine 14, 235-46.

Gore, A.C., Wu, T.J., Oung, T., Lee, J.B. and Woller, M.J. (2002) A novel mechanism for endocrine-disrupting effects of polychlorinated biphenyls: direct effects on gonadotropin-releasing hormone neurones. J Neuroendocrinol 14, 814-23.

Gore, A.C., Heindel, J.J. and Zoeller, R.T. (2006a) Endocrine disruption for endocrinologists (and others). Endocrinology 147, s1-s3.

Grabic, R., Hansen, L.G., Ptak, A., Crhova, S. and Gregoraszczuk, E.L. (2006) Differential accumulation of low-chlorinated (Delor 103) and high-chlorinated (Delor 106) biphenyls in human placental tissue and opposite effects on conversion of DHEA to E2. Chemosphere 62, 573-80.

Grasman, K.A., Scanlon, P.F. and Fox, G.A. (2000) Geographic variation in hematological variables in adult and prefledgling herring gulls (Larus argentatus) and possible associations with organochlorine exposure. Arch Environ Contam Toxicol 38, 244-53.

Gray, L.E., Ostby, J., Furr, J., Wolf, C.J., Lambright, C., Parks, L., Veeramachaneni, D.N., Wilson, V., Price, M., Hotchkiss, A., Orlando, E. and Guillette, L. (2001) Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update 7, 248-64.

Gray, L.E., Jr., Ostby, J.S. and Kelce, W.R. (1994) Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol Appl Pharmacol 129, 46-52.

Gregoraszczuk, E.L., Zemla, M., Ptak, A. and Grabic, R. (2005) The action of low- and high-chlorinated biphenyl mixture on prepubertal porcine ovary: steroid secretion and cells apoptosis. Endocr Regul 39, 33-41.

Gu, G.B. and Simerly, R.B. (1997) Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. J Comp Neurol 384, 142-64.

Gunderson, E.L. (1995) FDA Total Diet Study, July 1986-April 1991, dietary intakes of pesticides, selected elements, and other chemicals. J AOAC Int 78, 1353-63.

Guo, Y.L., Yu, M.L., Hsu, C.C. and Rogan, W.J. (1999) Chloracne, goiter, arthritis, and anemia after polychlorinated biphenyl poisoning: 14-year follow-Up of the Taiwan Yucheng cohort. Environ Health Perspect 107, 715-9.

Guo, Y.L., Hsu, P.C., Hsu, C.C. and Lambert, G.H. (2000) Semen quality after prenatal exposure to polychlorinated biphenyls and dibenzofurans. Lancet 356, 1240-1.

Guo, Y.L., Lambert, G.H., Hsu, C.C. and Hsu, M.M. (2004) Yucheng: health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. Int Arch Occup Environ Health 77, 153-8.

Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E. and Taipale, J. (2006) Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. Cell 124, 47-59.

Hammer, F., Compagnone, N.A., Vigne, J.L., Bair, S.R. and Mellon, S.H. (2004) Transcriptional regulation of P450scc gene expression in the embryonic rodent nervous system. Endocrinology 145, 901-12.

Han, J.S., Bird, G.C., Li, W., Jones, J. and Neugebauer, V. (2005) Computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior. J Neurosci Methods 141, 261-9.

Hanrahan, L.P., Falk, C., Anderson, H.A., Draheim, L., Kanarek, M.S. and Olson, J. (1999) Serum PCB and DDE levels of frequent Great Lakes sport fish consumers-a first look. The Great Lakes Consortium. Environ Res 80, S26-S37.

Hansen, L. Identification of steady state and episodic PCB congeners from multiple pathway exposures, Recent advances in the environmental toxicology and health effects of PCBs. Robertson, L. and Hansen, L., Lexington (2001) Pages.

Hany, J., Lilienthal, H., Sarasin, A., Roth-Harer, A., Fastabend, A., Dunemann, L., Lichtensteiger, W. and Winneke, G. (1999) Developmental exposure of rats to a reconstituted PCB mixture or aroclor 1254: effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. Toxicol Appl Pharmacol 158, 231-43.

Hao, P., He, W.Z., Huang, Y., Ma, L.X., Xu, Y., Xi, H., Wang, C., Liu, B.S., Wang, J.M., Li, Y.X. and Zhong, Y. (2005) MPSS: an integrated database system for surveying a set of proteins. Bioinformatics 21, 2142-3.

Hapon, M.B., Varas, S.M., Gimenez, M.S. and Jahn, G.A. (2007) Reduction of Mammary and Liver Lipogenesis and Alteration of Milk Composition during Lactation in Rats by Hypothyroidism. Thyroid 17, 11-8.

Hauser, R., Chen, Z., Pothier, L., Ryan, L. and Altshul, L. (2003) The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. Environ Health Perspect 111, 1505-11.

Hellou, J., Yeats, P., Steller, S. and Gagne, F. (2003) Chemical contaminants and biological indicators of mussel health during gametogenesis. Environ Toxicol Chem 22, 2080-7.

Herman, J.P. and Cullinan, W.E. (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20, 78-84.

Hermanson, M. and Hhites, R. (1989) Longterm measurements of atmospheric polychlorinated biphenyls in the vicinity of superfund dumps. Environmental Science and Technology 23, 1253-8.

Hernandez, J.P., Chapman, L.M., Kretschmer, X.C. and Baldwin, W.S. (2006) Gender-specific induction of cytochrome P450s in nonylphenol-treated FVB/NJ mice. Toxicol Appl Pharmacol 216, 186-96.

Hertz-Picciotto, I., James-Baker, R., Charles, M.D. and Greenfield, T. (2003) PCB exposures and sex ratio. Epidemiology 14, S89.

Ho, A. and Sudhof, T.C. (2004) Binding of F-spondin to amyloid-beta precursor protein: a candidate amyloid-beta precursor protein ligand that modulates amyloid-beta precursor protein cleavage. Proc Natl Acad Sci U S A 101, 2548-53.

Hodgert Jury, H., Zacharewski, T.R. and Hammond, G.L. (2000) Interactions between human plasma sex hormone-binding globulin and xenobiotic ligands. J Steroid Biochem Mol Biol 75, 167-76.

Hoepfner, B.A. and Ward, I.L. (1988) Prenatal and neonatal androgen exposure interact to affect sexual differentiation in female rats. Behav Neurosci 102, 61-5.

Holene, E., Nafstad, I., Skaare, J.U., Bernhoft, A., Engen, P. and Sagvolden, T. (1995) Behavioral-effects of prenatal and postnatal exposure to individual polychlorinated biphenyl congeners in rats. ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY 14, 967-76.

Holene, E., Nafstad, I., Skaare, J.U. and Sagvolden, T. (1998) Behavioural hyperactivity in rats following postnatal exposure to sub-toxic doses of polychlorinated biphenyl congeners 153 and 126. Behav Brain Res 94, 213-24.

Holloway, A.C., Kellenberger, L.D. and Petrik, J.J. (2006) Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. Endocrine 30, 213-6.

Horio, F. and Yoshida, A. (1982) Effects of some xenobiotics on ascorbic acid metabolism in rats. J Nutr 112, 416-25.

Howard, A.S., Fitzpatrick, R., Pessah, I., Kostyniak, P. and Lein, P.J. (2003) Polychlorinated biphenyls induce caspase-dependent cell death in cultured embryonic rat hippocampal but not cortical neurons via activation of the ryanodine receptor. Toxicol Appl Pharmacol 190, 72-86.

Iglewicz, B. and Hoaglin, D.C. (1993) How to detect and handle outliers. Journal 16,
Ihle, J.N. and Askew, D. (1989) Origins and properties of hematopoietic growth factor-dependent cell lines. Int J Cell Cloning 7, 68-91.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama, S. and Nishide, E. (1976) Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J Nutr Sci Vitaminol (Tokyo) 22, 409-18.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B. and Speed, T.P. (2003a) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31, e15.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. and Speed, T.P. (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249-64.

Jacobson, J.L. and Jacobson, S.W. (1997) Evidence for PCBs as neurodevelopmental toxicants in humans. Neurotoxicology 18, 415-24.

Jahn, O., Radulovic, J., Stiedl, O., Tezval, H., Eckart, K. and Spiess, J. (2005) Corticotropin-releasing factor binding protein--a ligand trap? Mini Rev Med Chem 5, 953-60.

Jansen, H.T., Cooke, P.S., Porcelli, J., Liu, T.C. and Hansen, L.G. (1993) Estrogenic and antiestrogenic actions of PCBs in the female rat: in vitro and in vivo studies. Reprod Toxicol 7, 237-48.

Jelaso, A.M., Lehigh-Shirey, E., Predenkiewicz, A., Means, J. and Ide, C.F. (2002) Aroclor 1254 alters morphology, survival, and gene expression in Xenopus laevis tadpoles. Environ Mol Mutagen 40, 24-35.

Jelaso, A.M., Lehigh-Shirey, E., Means, J. and Ide, C.F. (2003) Gene expression patterns predict exposure to PCBs in developing Xenopus laevis tadpoles. Environ Mol Mutagen 42, 1-10.

Jenkins, W.J. and Becker, J.B. (2003) Dynamic increases in dopamine during paced copulation in the female rat. Eur J Neurosci 18, 1997-2001.

Jensen, K.G., Wiberg, K., Klasson-Wehler, E. and Onfelt, A. (2000) Induction of aberrant mitosis with PCBs: particular efficiency of 2, 3,3',4,4'-pentachlorobiphenyl and synergism with triphenyltin. Mutagenesis 15, 9-15.

Johansson, M., Larsson, C., Bergman, A. and Lund, B.O. (1998a) Structure-activity relationship for inhibition of CYP11B1-dependent glucocorticoid synthesis in Y1 cells by aryl methyl sulfones. Pharmacol Toxicol 83, 225-30.

Johansson, M., Nilsson, S. and Lund, B.O. (1998b) Interactions between methylsulfonyl PCBs and the glucocorticoid receptor. Environ Health Perspect 106, 769-72.

Johansson, P.A., Dziegielewska, K.M., Ek, C.J., Habgood, M.D., Liddelow, S.A., Potter, A.M., Stolp, H.B. and Saunders, N.R. (2006) Blood-CSF barrier function in the rat embryo. Eur J Neurosci 24, 65-76.

Jourdan, D., Ardid, D., Chapuy, E., Eschalier, A. and Le Bars, D. (1995) Audible and ultrasonic vocalization elicited by single electrical nociceptive stimuli to the tail in the rat. Pain 63, 237-49.

Jourdan, D., Ardid, D., Chapuy, E., Le Bars, D. and Eschalier, A. (1997) Audible and ultrasonic vocalization elicited by a nociceptive stimulus in rat: relationship with respiration. J Pharmacol Toxicol Methods 38, 109-16.

Jourdan, D., Ardid, D., Chapuy, E., Le Bars, D. and Eschalier, A. (1998) Effect of analgesics on audible and ultrasonic pain-induced vocalization in the rat. Life Sci 63, 1761-8.

Juarez De Ku, L.M., Sharma-Stokkermans, M. and Meserve, L.A. (1994) Thyroxine normalizes polychlorinated biphenyl (PCB) dose-related depression of choline acetyltransferase (ChAT) activity in hippocampus and basal forebrain of 15-day-old rats. Toxicology 94, 19-30.

Kafafi, S.A., Afeefy, H.Y., Ali, A.H., Said, H.K. and Kafafi, A.G. (1993) Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. Environ Health Perspect 101, 422-8.

Kaltwasser, M.T. (1990) Startle-inducing acoustic stimuli evoke ultrasonic vocalization in the rat. Physiol Behav 48, 13-7.

Kaneko, T., Yoshimoto, H., Horiuchi, S. and Ikeda, Y. (1974) Proceedings: Effects of PCB administration on two generations and its chronic toxicity on the third generation in mice. 2. Jpn J Pharmacol 24, s:44.

Kang, J.H., Jeong, W., Park, Y., Lee, S.Y., Chung, M.W., Lim, H.K., Park, I.S., Choi, K.H., Chung, S.Y., Kim, D.S., Park, C.S., Hwang, O. and Kim, J. (2002) Aroclor 1254-induced cytotoxicity in catecholaminergic CATH.a cells related to the inhibition of NO production. Toxicology 177, 157-66.

Kato, N., Mochizuki, S., Kawai, K. and Yoshida, A. (1982) Effect of dietary level of sulfur-containing amino acids on liver drug-metabolizing enzymes, serum cholesterol and urinary ascorbic acid in rats fed PCB. J Nutr 112, 848-54.

Kaya, H., Hany, J., Fastabend, A., Roth-Harer, A., Winneke, G. and Lilienthal, H. (2002) Effects of maternal exposure to a reconstituted mixture of polychlorinated biphenyls on sex-dependent behaviors and steroid hormone concentrations in rats: dose-response relationship. Toxicol Appl Pharmacol 178, 71-81.

Kayser, V., Berkley, K.J., Keita, H., Gautron, M. and Guilbaud, G. (1996) Estrous and sex variations in vocalization thresholds to hindpaw and tail pressure stimulation in the rat. Brain Res 742, 352-4.

Kerkvliet, N.I., Baecher-Steppan, L., Smith, B.B., Youngberg, J.A., Henderson, M.C. and Buhler, D.R. (1990) Role of the Ah locus in suppression of cytotoxic T lymphocyte activity by halogenated aromatic hydrocarbons (PCBs and TCDD):

structure-activity relationships and effects in C57Bl/6 mice congenic at the Ah locus. Fundam Appl Toxicol 14, 532-41.

Kerr, M.K., Martin, M. and Churchill, G.A. (2000) Analysis of variance for gene expression microarray data. J Comput Biol 7, 819-37.

Kerr, M.K. and Churchill, G.A. (2001) Statistical design and the analysis of gene expression microarray data. Genet Res 77, 123-8.

Kester, M.H., Bulduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C.N., Coughtrie, M.W., Bergman, A., Safe, S.H., Kuiper, G.G., Schuur, A.G., Brouwer, A. and Visser, T.J. (2000) Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. Endocrinology 141, 1897-900.

Khan, I.A. and Thomas, P. (1997) Aroclor 1254-induced alterations in hypothalamic monoamine metabolism in the Atlantic croaker (Micropogonias undulatas): correlation with pituitary gonadotropin release. Neurotoxicology 18, 553-60.

Khan, I.A. and Thomas, P. (2000) Lead and Aroclor 1254 disrupt reproductive neuroendocrine function in Atlantic croaker. Mar Environ Res 50, 119-23.

Khan, I.A. and Thomas, P. (2001) Disruption of neuroendocrine control of luteinizing hormone secretion by aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. Biol Reprod 64, 955-64.

Khan, M.A., Lichtensteiger, C.A., Faroon, O., Mumtaz, M., Schaeffer, D.J. and Hansen, L.G. (2002) The hypothalamo-pituitary-thyroid (HPT) axis: a target of nonpersistent ortho-substituted PCB congeners. Toxicol Sci 65, 52-61.

Kholkute, S.D., Rodriguez, J. and Dukelow, W.R. (1994a) Reproductive toxicity of Aroclor-1254: effects on oocyte, spermatozoa, in vitro fertilization, and embryo development in the mouse. Reprod Toxicol 8, 487-93.

Kholkute, S.D., Rodriguez, J. and Dukelow, W.R. (1994b) Effects of polychlorinated biphenyls (PCBs) on in vitro fertilization in the mouse. Reprod Toxicol 8, 69-73.

Kilic, N., Sandal, S., Colakoglu, N., Kutlu, S., Seyran, A. and Yilmaz, B. (2005) Endocrine disruptive effects of polychlorinated biphenyls on the thyroid gland in female rats. Tohoku J Exp Med 206, 327-32.

Kimura-Kuroda, J., Nagata, I. and Kuroda, Y. (2005) Hydroxylated metabolites of polychlorinated biphenyls inhibit thyroid-hormone-dependent extension of cerebellar Purkinje cell dendrites. Brain Res Dev Brain Res 154, 259-63.

Kishimoto, T., Radulovic, J., Radulovic, M., Lin, C.R., Schrick, C., Hooshmand, F., Hermanson, O., Rosenfeld, M.G. and Spiess, J. (2000) Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet 24, 415-9.

Klinge, C.M., Bowers, J.L., Kulakosky, P.C., Kamboj, K.K. and Swanson, H.I. (1999) The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. Mol Cell Endocrinol 157, 105-19.

Kodavanti, P.R., Derr-Yellin, E.C., Mundy, W.R., Shafer, T.J., Herr, D.W., Barone, S., Choksi, N.Y., Macphail, R.C. and Tilson, H.A. (1998) Repeated exposure of adult rats to Aroclor 1254 causes brain region-specific changes in intracellular Ca2+ buffering and protein kinase C activity in the absence of changes in tyrosine hydroxylase. Toxicol Appl Pharmacol 153, 186-98.

Kohn, M.C. and Melnick, R.L. (2002) Biochemical origins of the non-monotonic receptor-mediated dose-response. J Mol Endocrinol 29, 113-23.

Kramer-Hammerle, S., Hahn, A., Brack-Werner, R. and Werner, T. (2005) Elucidating effects of long-term expression of HIV-1 Nef on astrocytes by microarray, promoter, and literature analyses. Gene 358, 31-8.

Krause, D.N., Duckles, S.P. and Pelligrino, D.A. (2006) Influence of sex steroid hormones on cerebrovascular function. J Appl Physiol 101, 1252-61.

Kremer, H., Lilienthal, H., Hany, J., Roth-Harer, A. and Winneke, G. (1999) Sex-dependent effects of maternal PCB exposure on the electroretinogram in adult rats. Neurotoxicol Teratol 21, 13-9.

Kuriyama, S.N. and Chahoud, I. (2004) In utero exposure to low-dose 2,3',4,4',5-pentachlorobiphenyl (PCB 118) impairs male fertility and alters neurobehavior in rat offspring. Toxicology 202, 185-97.

Lackmann, G.M. (2002) Polychlorinated biphenyls and hexachlorobenzene in full-term neonates. Reference values updated. Biol Neonate 81, 82-5.

Lai, T.J., Guo, Y.L., Yu, M.L., Ko, H.C. and Hsu, C.C. (1994) Cognitive development in Yucheng children. Chemosphere 29, 2405-11.

Landrigan, P.J., Sonawane, B., Butler, R.N., Trasande, L., Callan, R. and Droller, D. (2005) Early environmental origins of neurodegenerative disease in later life. Environ Health Perspect 113, 1230-3.

Langer, P., Kocan, A., Tajtakova, M., Petrik, J., Chovancova, J., Drobna, B., Jursa, S., Pavuk, M., Koska, J., Trnovec, T., Sebokova, E. and Klimes, I. (2003) Possible effects of polychlorinated biphenyls and organochlorinated pesticides on the thyroid after long-term exposure to heavy environmental pollution. J Occup Environ Med 45, 526-32.

Lasky, R.E., Widholm, J.J., Crofton, K.M. and Schantz, S.L. (2002) Perinatal exposure to Aroclor 1254 impairs distortion product otoacoustic emissions (DPOAEs) in rats. Toxicol Sci 68, 458-64.

Layton, A.C., Sanseverino, J., Gregory, B.W., Easter, J.P., Sayler, G.S. and Schultz, T.W. (2002) In vitro estrogen receptor binding of PCBs: measured activity and detection of hydroxylated metabolites in a recombinant yeast assay. Toxicol Appl Pharmacol 180, 157-63.

Lebel, M., Gauthier, Y., Moreau, A. and Drouin, J. (2001) Pitx3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site. J Neurochem 77, 558-67.

Lee, C.K., Lee, C.U., Kim, J.H., Son, B.C., Kim, D.H., Lee, C.H., Kim, H.D., Kim, J.W., Yoon, Y.D., Kang, S.G. and Moon, D.H. (2003) Effects of Aroclor 1254 on the expression of rat placental PRL-family genes. Mol Cells 15, 114-21.

Lee, D.W., Gelein, R.M. and Opanashuk, L.A. (2006) Heme-oxygenase-1 promotes polychlorinated biphenyl mixture aroclor 1254-induced oxidative stress and dopaminergic cell injury. Toxicol Sci 90, 159-67.

Lelievre, E., Lionneton, F., Soncin, F. and Vandenbunder, B. (2001) The Ets family contains transcriptional activators and repressors involved in angiogenesis. Int J Biochem Cell Biol 33, 391-407.

Levin, M., De Guise, S. and Ross, P.S. (2005) Association between lymphocyte proliferation and polychlorinated biphenyls in free-ranging harbor seal (Phoca vitulina) pups from British Columbia, Canada. Environ Toxicol Chem 24, 1247-52.

Levine, J.D., Feldmesser, M., Tecott, L., Gordon, N.C. and Izdebski, K. (1984) Pain-induced vocalization in the rat and its modification by pharmacological agents. Brain Res 296, 121-7.

Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P.E. and Vale, W.W. (2001) Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc Natl Acad Sci U S A 98, 7570-5.

Li, L.A., Wang, P.W. and Chang, L.W. (2004) Polychlorinated biphenyl 126 stimulates basal and inducible aldosterone biosynthesis of human adrenocortical H295R cells. Toxicol Appl Pharmacol 195, 92-102.

Li, L.A. and Wang, P.W. (2005) PCB126 induces differential changes in androgen, cortisol, and aldosterone biosynthesis in human adrenocortical H295R cells. Toxicol Sci 85, 530-40.

Li, S., Washburn, K.A., Moore, R., Uno, T., Teng, C., Newbold, R.R., Mclachlan, J.A. and Negishi, M. (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57, 4356-9.

Li, X., Zhang, S. and Safe, S. (2006) Activation of kinase pathways in MCF-7 cells by 17beta-estradiol and structurally diverse estrogenic compounds. J Steroid Biochem Mol Biol 98, 122-32.

Li, Y., Millikan, R.C., Bell, D.A., Cui, L., Tse, C.K., Newman, B. and Conway, K. (2005) Polychlorinated biphenyls, cytochrome P450 1A1 (CYP1A1) polymorphisms, and breast cancer risk among African American women and white women in North Carolina: a population-based case-control study. Breast Cancer Res 7, R12-8.

Lilienthal, H., Neuf, M., Munoz, C. and Winneke, G. (1990) Behavioral effects of pre- and postnatal exposure to a mixture of low chlorinated PCBs in rats. Fundam Appl Toxicol 15, 457-67.

Lilienthal, H., Weinand-Harer, A., Winterhoff, H. and Winneke, G. (1997) Effects of maternal exposure to 3,3',4,4'-tetrachlorobiphenyl or propylthiouracil in rats trained to discriminate apomorphine from saline. Toxicol Appl Pharmacol 146, 162-9.

Lilienthal, H., Fastabend, A., Hany, J., Kaya, H., Roth-Harer, A., Dunemann, L. and Winneke, G. (2000) Reduced levels of 1,25-dihydroxyvitamin D(3) in rat dams and offspring after exposure to a reconstituted PCB mixture. Toxicol Sci 57, 292-301.

Liljegren, G., Hardell, L., Lindstrom, G., Dahl, P. and Magnuson, A. (1998) Case-control study on breast cancer and adipose tissue concentrations of congener specific polychlorinated biphenyls, DDE and hexachlorobenzene. Eur J Cancer Prev 7, 135-40.

Lin, T.C., Chien, S.C., Hsu, P.C. and Li, L.A. (2006a) Mechanistic study of polychlorinated biphenyl 126-induced CYP11B1 and CYP11B2 up-regulation. Endocrinology 147, 1536-44.

Lin, T.C., Chien, S.C., Hsu, P.C. and Li, L.A. (2006b) Mechanistic study of polychlorinated biphenyl 126-induced CYP11B1 and CYP11B2 up-regulation. Endocrinology 147, 1536-44.

Littell, R.C., Milliken, G.A., Stroup, W.W. and Wolfinger, R.D. SAS systems for mixed models, Cary, NC (1999) Pages.

Liu, J., Liu, Y., Barter, R.A. and Klaassen, C.D. (1995) Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. J Pharmacol Exp Ther 273, 977-85.

Loch-Caruso, R. (2002) Uterine muscle as a potential target of polychlorinated biphenyls during pregnancy. Int J Hyg Environ Health 205, 121-30.

Lorenzen, A., Moon, T.W., Kennedy, S.W. and Glen, G.A. (1999) Relationships between environmental organochlorine contaminant residues, plasma corticosterone concentrations, and intermediary metabolic enzyme activities in Great Lakes herring gull embryos. Environ Health Perspect 107, 179-86.

Love, O.P., Shutt, L.J., Silfies, J.S., Bortolotti, G.R., Smits, J.E. and Bird, D.M. (2003) Effects of dietary PCB exposure on adrenocortical function in captive American kestrels (Falco sparverius). Ecotoxicology 12, 199-208.

Lucena, R.A., Allam, M.F., Costabeber, I.H., Villarejo, M.L. and Navajas, R.F. (2001) Breast cancer risk factors: PCB congeners. Eur J Cancer Prev 10, 117-9.

Lyche, J., Larsen, H., Skaare, J.U., Tverdal, A., Dahl, E., Johansen, G. and Ropstad, E. (2004) Effects of perinatal exposure to low doses of PCB 153 and PCB 126 on lymphocyte proliferation and hematology in goat kids. J Toxicol Environ Health A 67, 889-904.

Lyche, J.L., Larsen, H.J., Utne Skaare, J., Tverdal, A., Johansen, G.M. and Ropstad, E. (2006) Perinatal exposure to low doses of PCB 153 and PCB 126 affects maternal and neonatal immunityin goat kids. J Toxicol Environ Health A 69, 139-58.

Machala, M., Blaha, L., Lehmler, H.J., Pliskova, M., Majkova, Z., Kapplova, P., Sovadinova, I., Vondracek, J., Malmberg, T. and Robertson, L.W. (2004) Toxicity of hydroxylated and quinoid PCB metabolites: inhibition of gap junctional intercellular communication and activation of aryl hydrocarbon and estrogen receptors in hepatic and mammary cells. Chem Res Toxicol 17, 340-7.

Mackenzie, C.A., Lockridge, A. and Keith, M. (2005) Declining Sex Ratio in a First Nation Community. Environmental Health Perspectives 113, 1295-8.

Maier, W.E., Kodavanti, P.R., Harry, G.J. and Tilson, H.A. (1994) Sensitivity of adenosine triphosphatases in different brain regions to polychlorinated biphenyl congeners. J Appl Toxicol 14, 225-9.

Maliwal, B.P. and Guthrie, F.E. (1982) In vitro uptake and transfer of chlorinated hydrocarbons among human lipoproteins. J Lipid Res 23, 474-9.

Malkiewicz, K., Mohammed, R., Folkesson, R., Winblad, B., Szutowski, M. and Benedikz, E. (2006) Polychlorinated biphenyls alter expression of alpha-synuclein, synaptophysin and parkin in the rat brain. Toxicol Lett 161, 152-8.

Mariussen, E., Andersson, P.L., Tysklind, M. and Fonnum, F. (2001) Effect of polychlorinated biphenyls on the uptake of dopamine into rat brain synaptic vesicles: a structure-activity study. Toxicol Appl Pharmacol 175, 176-83.

Mariussen, E. and Fonnum, F. (2001) The effect of polychlorinated biphenyls on the high affinity uptake of the neurotransmitters, dopamine, serotonin, glutamate and GABA, into rat brain synaptosomes. Toxicology 159, 11-21.

Mariussen, E., Myhre, O., Reistad, T. and Fonnum, F. (2002) The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: the involvement of the N-methyl-D-aspartate receptor and reactive oxygen species. Toxicol Appl Pharmacol 179, 137-44.

Marois, G. (1968) [Action of progesterone, testosterone and estradiol on the anogenital distance and somatic sexual differentiation in rats]. Biol Med (Paris) 57, 44-90.

Masuda, Y. (2001) Fate of PCDF/PCB congeners and change of clinical symptoms in patients with Yusho PCB poisoning for 30 years. Chemosphere 43, 925-30.

Matthews, H. and Anderson, M. (1975) Effect of Chlorination on the Distribution and Excretion of Polychlorinated Biphenyls. Metabolism and Disposition 3, 371-80.

Mcclintock, M. (1983) The Behavioral Endocrinology of Rodents: A Functional Analysis. BioScience 33, 573-7.

Mcconnell, E.E., Hass, J.R., Altman, N. and Moore, J.A. (1979) A spontaneous outbreak of polycholorinated biphenyl (PCB) toxicity in rhesus monkeys (Macaca mulatta): toxicopathology. Lab Anim Sci 29, 666-73.

Mccormick, C.M., Linkroum, W., Sallinen, B.J. and Miller, N.W. (2002) Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. Stress 5, 235-47.

Mccoy, G., Finlay, M.F., Rhone, A., James, K. and Cobb, G.P. (1995) Chronic polychlorinated biphenyls exposure on three generations of oldfield mice (Peromyscus polionotus): effects on reproduction, growth, and body residues. Arch Environ Contam Toxicol 28, 431-5.

Mcdonald, A.J., Mascagni, F. and Wilson, M.A. (1994) A sexually dimorphic population of CRF neurons in the medial preoptic area. Neuroreport 5, 653-6.

Mclean, M.R., Robertson, L.W. and Gupta, R.C. (1996) Detection of PCB adducts by the 32P-postlabeling technique. Chem Res Toxicol 9, 165-71.

Meaney, M.J. (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24, 1161-92.

Medlock, K.L., Lyttle, C.R., Kelepouris, N., Newman, E.D. and Sheehan, D.M. (1991) Estradiol down-regulation of the rat uterine estrogen receptor. Proc Soc Exp Biol Med 196, 293-300.

Medlock, K.L., Forrester, T.M. and Sheehan, D.M. (1994) Progesterone and estradiol interaction in the regulation of rat uterine weight and estrogen receptor concentration. Proc Soc Exp Biol Med 205, 146-53.

Meeks, T.W., Ropacki, S.A. and Jeste, D.V. (2006) The neurobiology of neuropsychiatric syndromes in dementia. Curr Opin Psychiatry 19, 581-6.

Meerts, H.A., Lilienthal, H., Hoving, S., Vand Den Berh, J.H., Weijers, B.M., Bergman, A., Koeman, J.H. and Brouwer, A. (2004a) Developmental exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107): Long-Term Effects on Brain

Development, Behavior, and Brain Stem Auditory Evoked Potential in Rats. Toxciological Sciences 82, 207-18.

Meerts, I.A., Hoving, S., Van Den Berg, J.H., Weijers, B.M., Swarts, H.J., Van Der Beek, E.M., Bergman, A., Koeman, J.H. and Brouwer, A. (2004b) Effects of in utero exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107) on developmental landmarks, steroid hormone levels, and female estrous cyclicity in rats. Toxicol Sci 82, 259-67.

Meerts, I.A., Lilienthal, H., Hoving, S., Van Den Berg, J.H., Weijers, B.M., Bergman, A., Koeman, J.H. and Brouwer, A. (2004c) Developmental exposure to 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107): long-term effects on brain development, behavior, and brain stem auditory evoked potentials in rats. Toxicol Sci 82, 207-18.

Miller, D.B., Gray, L.E., Jr., Andrews, J.E., Luebke, R.W. and Smialowicz, R.J. (1993) Repeated exposure to the polychlorinated biphenyl (Aroclor 1254) elevates the basal serum levels of corticosterone but does not affect the stress-induced rise. Toxicology 81, 217-22.

Mlynarczuk, J. and Kotwica, J. (2005) Influence of polychlorinated biphenyles on the secretion of oxytocin from bovine luteal cells and from granulosa cells obtained from the follicles of different size. Pol J Vet Sci 8, 261-7.

Mol, N.M., Sorensen, N., Weihe, P., Andersson, A.M., Jorgensen, N., Skakkebaek, N.E., Keiding, N. and Grandjean, P. (2002) Spermaturia and serum hormone concentrations at the age of puberty in boys prenatally exposed to polychlorinated biphenyls. Eur J Endocrinol 146, 357-63.

Moore, C. and Morelli, G. (1979) Mother rats interact differently with male and female offspring. J Comp Physiol Pychol 93, 677-84.

Moore, M., Mustain, M., Daniel, K., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A. and Balaguer, P. (1997) Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. Toxicol Appl Pharmacol 142, 160-8.

Morse, D.C., Plug, A., Wesseling, W., Van Den Berg, K.J. and Brouwer, A. (1996a) Persistent alterations in regional brain glial fibrillay acidic protein and synaptotagmin levels following pre- and postnatal polychlorinated biphenyl exposure. Toxicol Appl Pharmacol 139, 252-61.

Morse, D.C., Seegal, R.F., Borsch, K.O. and Brouwer, A. (1996b) Long-term alterations in regional brain serotonin metabolism following maternal polychlorinated biphenyl exposure in the rat. Neurotoxicology 17, 631-8.

Morse, D.C., Wehler, E.K., Wesseling, W., Koeman, J.H. and Brouwer, A. (1996c) Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). Toxicol Appl Pharmacol 136, 269-79.

Moysich, K.B., Ambrosone, C.B., Vena, J.E., Shields, P.G., Mendola, P., Kostyniak, P., Greizerstein, H., Graham, S., Marshall, J.R., Schisterman, E.F. and Freudenheim, J.L. (1998) Environmental organochlorine exposure and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 7, 181-8.

Muir, D.C., Ford, C.A., Rosenberg, B., Norstrom, R.J., Simon, M. and Beland, P. (1996) Persistent organochlorines in beluga whales (Delphinapterus leucas) from the St Lawrence River estuary--I. Concentrations and patterns of specific PCBs, chlorinated pesticides and polychlorinated dibenzo-p-dioxins and dibenzofurans. Environ Pollut 93, 219-34.

Murugesan, P., Kanagaraj, P., Yuvaraj, S., Balasubramanian, K., Aruldhas, M.M. and Arunakaran, J. (2005) The inhibitory effects of polychlorinated biphenyl Aroclor 1254 on Leydig cell LH receptors, steroidogenic enzymes and antioxidant enzymes in adult rats. Reprod Toxicol 20, 117-26.

Muthuvel, R., Venkataraman, P., Krishnamoorthy, G., Gunadharini, D.N., Kanagaraj, P., Jone Stanley, A., Srinivasan, N., Balasubramanian, K., Aruldhas, M.M. and Arunakaran, J. (2006) Antioxidant effect of ascorbic acid on PCB (Aroclor 1254) induced oxidative stress in hypothalamus of albino rats. Clin Chim Acta 365, 297-303.

Nesaretnam, K., Corcoran, D., Dils, R.R. and Darbre, P. (1996) 3,4,3',4'-Tetrachlorobiphenyl acts as an estrogen in vitro and in vivo. Mol Endocrinol 10, 923-36.

Newbold, R.R., Hanson, R.B., Jefferson, W.N., Bullock, B.C., Haseman, J. and Mclachlan, J.A. (1998) Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis 19, 1655-63.

Newbold, R.R., Hanson, R.B., Jefferson, W.N., Bullock, B.C., Haseman, J. and Mclachlan, J.A. (2000) Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis 21, 1355-63.

Newbold, R.R., Padilla-Banks, E. and Jefferson, W.N. (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 147, S11-7.

Nilsson, C.B., Hoegberg, P., Trossvik, C., Azais-Braesco, V., Blaner, W.S., Fex, G., Harrison, E.H., Nau, H., Schmidt, C.K., Van Bennekum, A.M. and Hakansson, H. (2000) 2,3,7,8-tetrachlorodibenzo-p-dioxin increases serum and kidney retinoic acid levels and kidney retinol esterification in the rat. Toxicol Appl Pharmacol 169, 121-31.

Nishida, W., Nakamura, M., Mori, S., Takahashi, M., Ohkawa, Y., Tadokoro, S., Yoshida, K., Hiwada, K., Hayashi, K. and Sobue, K. (2002) A triad of serum response factor and the GATA and NK families governs the transcription of smooth and cardiac muscle genes. J Biol Chem 277, 7308-17.

Nishihara, Y. and Utsumi, K. (1987) 4-Chloro-4'-biphenylol as an uncoupler and an inhibitor of mitochondrial oxidative phosphorylation. Biochem Pharmacol 36, 3453-7.

Nixon, A., Benghuzzi, H. and Cason, Z. (2003) Morphometric evaluation of ovarian tissue exposed to PCB conventionally and in a sustained manner. Biomed Sci Instrum 39, 434-9.

(2006) National Toxicology Program toxicology and carcinogenesis studies of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) (CAS No. 57465-28-8) in female Harlan Sprague-Dawley rats (Gavage Studies). 4-246.

Ohtsubo, Y., Kudo, T., Tsuda, M. and Nagata, Y. (2004) Strategies for bioremediation of polychlorinated biphenyls. Appl Microbiol Biotechnol 65, 250-8.

Oskam, I., Ropstad, E., Lie, E., Derocher, A., Wiig, O., Dahl, E., Larsen, S. and Skaare, J.U. (2004) Organochlorines affect the steroid hormone cortisol in free-ranging polar bears (Ursus maritimus) at Svalbard, Norway. J Toxicol Environ Health A 67, 959-77.

Palanza, P.L., Howdeshell, K.L., Parmigiani, S. and Vom Saal, F.S. (2002) Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ Health Perspect 110 Suppl 3, 415-22.

Paredes, R.G. and Vazquez, B. (1999) What do female rats like about sex? Paced mating. Behav Brain Res 105, 117-27.

Pasterkamp, R.J., Yuri, K., Visser, D.T., Hayashi, S. and Kawata, M. (1996) The perinatal ontogeny of estrogen receptor-immunoreactivity in the developing male and female rat hypothalamus. Brain Res Dev Brain Res 91, 300-3.

Patisaul, H.B., Fortino, A.E. and Polston, E.K. (2006) Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicol Teratol 28, 111-8.

Patnode, K.A. and Curtis, L.R. (1994) 2,2',4,4',5,5'- and 3,3',4,4',5,5'-hexachlorobiphenyl alteration of uterine progesterone and estrogen receptors coincides with embryotoxicity in mink (Mustela vision). Toxicol Appl Pharmacol 127, 9-18.

Pavuk, M., Cerhan, J.R., Lynch, C.F., Schecter, A., Petrik, J., Chovancova, J. and Kocan, A. (2004) Environmental exposure to PCBs and cancer incidence in eastern Slovakia. Chemosphere 54, 1509-20.

Pawllkowski, M., Grochal, M., Kulig, A., Zielinski, K., Stepien, H., Kunert-Radek, J. and Mucha, S. (1996) The effect of angiotensin II receptor antagonists on diethylstilbestrol-induced vascular changes in the rat anterior pituitary gland: a quantitative evaluation. Histol Histopathol 11, 909-13.

Pearce, S.T., Liu, H., Radhakrishnan, I., Abdelrahim, M., Safe, S. and Jordan, V.C. (2004) Interaction of the aryl hydrocarbon receptor ligand 6-methyl-1,3,8-trichlorodibenzofuran with estrogen receptor alpha. Cancer Res 64, 2889-97.

Pei, H., Li, C., Adereth, Y., Hsu, T., Watson, D.K. and Li, R. (2005) Caspase-1 is a direct target gene of ETS1 and plays a role in ETS1-induced apoptosis. Cancer Res 65, 7205-13.

Pellis, S.M. and Pellis, V.C. (1997) The prejuvenile onset of play fighting in laboratory rats (Rattus norvegicus). Dev Psychobiol 31, 193-205.

Perakis, A. and Stylianopoulou, F. (1986) Effects of a prenatal androgen peak on rat brain sexual differentiation. J Endocrinol 108, 281-5.

Peruzzo, B., Pastor, F.E., Blazquez, J.L., Schobitz, K., Pelaez, B., Amat, P. and Rodriguez, E.M. (2000) A second look at the barriers of the medial basal hypothalamus. Exp Brain Res 132, 10-26.

Petit, F., Le Goff, P., Cravedi, J.P., Valotaire, Y. and Pakdel, F. (1997) Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J Mol Endocrinol 19, 321-35.

Pillon, A., Boussioux, A.M., Escande, A., Ait-Aissa, S., Gomez, E., Fenet, H., Ruff, M., Moras, D., Vignon, F., Duchesne, M.J., Casellas, C., Nicolas, J.C. and Balaguer, P. (2005) Binding of estrogenic compounds to recombinant estrogen receptor-alpha: application to environmental analysis. Environ Health Perspect 113, 278-84.

Platonow, N.S. and Meads, E.B. (1975) Distribution and excretion of two chlorinated biphenyl isomers: 4-chlorobiphenyl and decachlorobiphenyl in lactating bovine. Can J Comp Med 39, 104-6.

Pocar, P., Brevini, T.A., Perazzoli, F., Cillo, F., Modina, S. and Gandolfi, F. (2001) Cellular and molecular mechanisms mediating the effects of polychlorinated biphenyls on oocyte developmental competence in cattle. Mol Reprod Dev 60, 535-41.

Porterfield, S.P. (1994) Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ Health Perspect 102 Suppl 2, 125-30.

Porterfield, S.P. and Hendry, L.B. (1998) Impact of PCBs on thyroid hormone directed brain development. Toxicol Ind Health 14, 103-20.

Pravettoni, A., Colciago, A., Negri-Cesi, P., Villa, S. and Celotti, F. (2005) Ontogenetic development, sexual differentiation, and effects of Aroclor 1254 exposure on expression of the arylhydrocarbon receptor and of the arylhydrocarbon receptor nuclear translocator in the rat hypothalamus. Reprod Toxicol 20, 521-30.

Primus, R.J. and Kellogg, C.K. (1990) Gonadal hormones during puberty organize environment-related social interaction in the male rat. Horm Behav 24, 311-23.

Prince, M.M., Ruder, A.M., Hein, M.J., Waters, M.A., Whelan, E.A., Nilsen, N., Ward, E.M., Schnorr, T.M., Laber, P.A. and Davis-King, K.E. (2006) Mortality and exposure

response among 14,458 electrical capacitor manufacturing workers exposed to polychlorinated biphenyls (PCBs). Environ Health Perspect 114, 1508-14.

Provost, T.L., Juarez De Ku, L.M., Zender, C. and Meserve, L.A. (1999a) Dose- and age-dependent alterations in choline acetyltransferase (ChAT) activity, learning and memory, and thyroid hormones in 15- and 30-day old rats exposed to 1.25 or 12.5 PPM polychlorinated biphenyl (PCB) beginning at conception. Prog Neuropsychopharmacol Biol Psychiatry 23, 915-28.

Provost, T.L., Juarez De Ku, L.M., Zender, C. and Meserve, L.A. (1999b) Dose- and age-dependent alterations in choline acetyltransferase (ChAT) activity, learning and memory, and thyroid hormones in 15- and 30-day old rats exposed to 1.25 or 12.5 PPM polychlorinated biphenyl (PCB) beginning at conception. Prog Neuropsychopharmacol Biol Psychiatry 23, 915-28.

Ptak, A., Ludewig, G., Lehmler, H.J., Wojtowicz, A.K., Robertson, L.W. and Gregoraszczuk, E.L. (2005) Comparison of the actions of 4-chlorobiphenyl and its hydroxylated metabolites on estradiol secretion by ovarian follicles in primary cells in culture. Reprod Toxicol 20, 57-64.

Ptak, A., Ludewig, G., Kapiszewska, M., Magnowska, Z., Lehmler, H.J., Robertson, L.W. and Gregoraszczuk, E.L. (2006a) Induction of cytochromes P450, caspase-3 and DNA damage by PCB3 and its hydroxylated metabolites in porcine ovary. Toxicol Lett 166, 200-11.

Ptak, A., Ludewig, G., Robertson, L., Lehmler, H.J. and Gregoraszczuk, E.L. (2006b) In vitro exposure of porcine prepubertal follicles to 4-chlorobiphenyl (PCB3) and its hydroxylated metabolites: effects on sex hormone levels and aromatase activity. Toxicol Lett 164, 113-22.

Purkey, H.E., Palaninathan, S.K., Kent, K.C., Smith, C., Safe, S.H., Sacchettini, J.C. and Kelly, J.W. (2004) Hydroxylated polychlorinated biphenyls selectively bind transthyretin in blood and inhibit amyloidogenesis: rationalizing rodent PCB toxicity. Chem Biol 11, 1719-28.

Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 23, 4878-84.

Rabadan-Diehl, C., Lolait, S. and Aguilera, G. (2000) Isolation and characterization of the promoter region of the rat vasopressin V1b receptor gene. J Neuroendocrinol 12, 437-44.

Ramaley, J.A. (1979) Development of Gonadotropin regulation in the prepubertal mammal. Biol Reprod 20, 1-31.

Ramamoorthy, K., Vyhlidal, C., Wang, F., Chen, I., Safe, S., Mcdonnell, D.P., Leonard, L.S. and Gaido, K.W. (1997) Additive estrogenic activities of a binary mixture of 2',4',6'-trichloro- and 2',3',4',5'-tetrachloro-4-biphenylol. Toxicol Appl Pharmacol 147, 93-100.

Raulf, M. and Konig, W. (1991) In vitro effects of polychlorinated biphenyls on human platelets. Immunology 72, 287-91.

Rehulka, J. and Minarik, B. (2004) Effect of polychlorinated biphenyls (Delor 103) on haematological and enzyme parameters of the rainbow trout Oncorhynchus mykiss. Dis Aquat Organ 62, 147-53.

Reyes, T.M., Lewis, K., Perrin, M.H., Kunitake, K.S., Vaughan, J., Arias, C.A., Hogenesch, J.B., Gulyas, J., Rivier, J., Vale, W.W. and Sawchenko, P.E. (2001) Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci U S A 98, 2843-8.

Rhees, R.W., Shryne, J.E. and Gorski, R.A. (1990) Onset of the hormone-sensitive perinatal period for sexual differentiation of the sexually dimorphic nucleus of the preoptic area in female rats. J Neurobiol 21, 781-6.

Richardson, J.R. and Miller, G.W. (2004) Acute exposure to aroclor 1016 or 1260 differentially affects dopamine transporter and vesicular monoamine transporter 2 levels. Toxicol Lett 148, 29-40.

Ritchie, J.M., Vial, S.L., Fuortes, L.J., Guo, H., Reedy, V.E. and Smith, E.M. (2003) Organochlorines and risk of prostate cancer. J Occup Environ Med 45, 692-702.

Roegge, C.S., Wang, V.C., Powers, B.E., Klintsova, A.Y., Villareal, S., Greenough, W.T. and Schantz, S.L. (2004) Motor impairment in rats exposed to PCBs and methylmercury during early development. Toxicol Sci 77, 315-24.

Roegge, C.S., Morris, J.R., Villareal, S., Wang, V.C., Powers, B.E., Klintsova, A.Y., Greenough, W.T., Pessah, I.N. and Schantz, S.L. (2006) Purkinje cell and cerebellar effects following developmental exposure to PCBs and/or MeHg. Neurotoxicol Teratol 28, 74-85.

Roelens, S.A., Beck, V., Aerts, G., Clerens, S., Vanden Bergh, G., Arckens, L., Darras, V.M. and S, V.D.G. (2005) Neurotoxicity of Polychlorinated Biphenyls (PCBs) by Disturbance of Thyroid Hormone-Regulated Genes. Ann N Y Acad Sci 1040, 454-6.

Romeo, R.D. (2003) Puberty: a period of both organizational and activational effects of steroid hormones on neurobehavioural development. J Neuroendocrinol 15, 1185-92.

Rosenblatt, J.S., Wagner, C.K. and Morrell, J.I. (1994) Hormonal priming and triggering of maternal behavior in the rat with special reference to the relations between estrogen receptor binding and ER mRNA in specific brain regions. Psychoneuroendocrinology 19, 543-52.

Ross, P.S., Van Loveren, H., De Swart, R.L., Van Der Vliet, H., De Klerk, A., Timmerman, H.H., Van Binnendijk, R., Brouwer, A., Vos, J.G. and Osterhaus, A.D.

(1996) Host resistance to rat cytomegalovirus (RCMV) and immune function in adult PVG rats fed herring from the contaminated Baltic Sea. Arch Toxicol 70, 661-71.

Roth-Harer, A., Lilienthal, H., Bubser, M., Kronthaler, U., W, R.M., T, R.W., Schmidt, W., Winterhoff, H. and Winneke, G. (2001) Neurotransmitter concentrations and binding at dopamine receptors in rats after maternal exposure to 3,4,3',4'-tetrachlorobiphenyl: the role of reduced thyroid hormone concentrations. Environ. Toxicol. Pharmacol. 9, 103-15.

Routledge, E.J., White, R., Parker, M.G. and Sumpter, J.P. (2000) Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 275, 35986-93.

Rozati, R., Reddy, P.P., Reddanna, P. and Mujtaba, R. (2002) Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 78, 1187-94.

Sager, D.B. and Girard, D.M. (1994) Long-term effects on reproductive parameters in female rats after translactational exposure to PCBs. Environ Res 66, 52-76.

Saito, M. (1990) Polychlorinated biphenyls-induced lipid peroxidation as measured by thiobarbituric acid-reactive substances in liver subcellular fractions of rats. Biochim Biophys Acta 1046, 301-8.

Salama, J., Chakraborty, T.R., Ng, L. and Gore, A.C. (2003) Effects of polychlorinated biphenyls on estrogen receptor-beta expression in the anteroventral periventricular nucleus. Environ Health Perspect 111, 1278-82.

Sargent, L., Dragan, Y.P., Erickson, C., Laufer, C.J. and Pitot, H.C. (1991) Study of the separate and combined effects of the non-planar 2,5,2',5'- and the planar 3,4,3',4'-tetrachlorobiphenyl in liver and lymphocytes in vivo. Carcinogenesis 12, 793-800.

Sawada, H. and Shimohama, S. (2003) Estrogens and Parkinson disease: novel approach for neuroprotection. Endocrine 21, 77-9.

Schaeffer, D.J., Dellinger, J.A., Needham, L.L. and Hansen, L.G. (2006) Serum PCB profiles in Native Americans from Wisconsin based on region, diet, age, and gender: Implications for epidemiology studies. Sci Total Environ 357, 74-87.

Schantz, S.L., Seo, B.W., Wong, P.W. and Pessah, I.N. (1997) Long-term effects of developmental exposure to 2,2',3,5',6-pentachlorobiphenyl (PCB 95) on locomotor activity, spatial learning and memory and brain ryanodine binding. Neurotoxicology 18, 457-67.

Schiestl, R.H., Aubrecht, J., Yap, W.Y., Kandikonda, S. and Sidhom, S. (1997) Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin induce intrachromosomal recombination in vitro and in vivo. Cancer Res 57, 4378-83.

Schrader, T.J. and Cooke, G.M. (2003) Effects of Aroclors and individual PCB congeners on activation of the human androgen receptor in vitro. Reprod Toxicol 17, 15-23.

Schwartz, N.B. (1974) The role of FSH and LH and of their antibodies on follicle growth and on ovulation. Biol Reprod 10, 236-72.

Seegal, R.F., Bush, B. and Brosch, K.O. (1985) Polychlorinated biphenyls induce regional changes in brain norepinephrine concentrations in adult rats. Neurotoxicology 6, 13-23.

Seegal, R.F., Brosch, K.O. and Bush, B. (1986) Regional alterations in serotonin metabolism induced by oral exposure of rats to polychlorinated biphenyls. Neurotoxicology 7, 155-65.

Seegal, R.F., Bush, B. and Shain, W. (1990) Lightly chlorinated ortho-substituted PCB congeners decrease dopamine in nonhuman primate brain and in tissue culture. Toxicol Appl Pharmacol 106, 136-44.

Seegal, R.F., Bush, B. and Brosch, K.O. (1991) Comparison of effects of Aroclors 1016 and 1260 on non-human primate catecholamine function. Toxicology 66, 145-63.

Seegal, R.F. (1994) The neurochemical effects of PCB exposure are age-dependent. Arch Toxicol Suppl 16, 128-37.

Seegal, R.F., Brosch, K.O. and Okoniewski, R.J. (1997) Effects of in utero and lactational exposure of the laboratory rat to 2,4,2',4'- and 3,4,3',4'-tetrachlorobiphenyl on dopamine function. Toxicol Appl Pharmacol 146, 95-103.

Seegal, R.F., Okoniewski, R.J., Brosch, K.O. and Bemis, J.C. (2002) Polychlorinated biphenyls alter extraneuronal but not tissue dopamine concentrations in adult rat striatum: an in vivo microdialysis study. Environ Health Perspect 110, 1113-7.

Seegal, R.F., Brosch, K.O. and Okoniewski, R.J. (2005) Coplanar PCB congeners increase uterine weight and frontal cortical dopamine in the developing rat: implications for developmental neurotoxicity. Toxicol Sci 86, 125-31.

Seo, H., Hong, S.J., Guo, S., Kim, H.S., Kim, C.H., Hwang, D.Y., Isacson, O., Rosenthal, A. and Kim, K.S. (2002) A direct role of the homeodomain proteins Phox2a/2b in noradrenaline neurotransmitter identity determination. J Neurochem 80, 905-16.

Shafer, T.J., Mundy, W.R., Tilson, H.A. and Kodavanti, P.R. (1996) Disruption of inositol phosphate accumulation in cerebellar granule cells by polychlorinated biphenyls: a consequence of altered Ca2+ homeostasis. Toxicol Appl Pharmacol 141, 448-55.

Shain, W., Bush, B. and Seegal, R. (1991) Neurotoxicity of polychlorinated biphenyls: structure-activity relationship of individual congeners. Toxicol Appl Pharmacol 111, 33-42.

Sharlin, D.S., Bansal, R. and Zoeller, R.T. (2006) Polychlorinated biphenyls exert selective effects on cellular composition of white matter in a manner inconsistent with thyroid hormone insufficiency. Endocrinology 147, 846-58.

Sharma, R. and Kodavanti, P.R. (2002) In vitro effects of polychlorinated biphenyls and hydroxy metabolites on nitric oxide synthases in rat brain. Toxicol Appl Pharmacol 178, 127-36.

Sharpe, R.M. (1975) The influence of the sex of litter-mates on subsequent maternal behaviour in Rattus norvegicus. Anim Behav 23, 551-9.

Shaw, S.D., Brenner, D., Berger, M.L., Carpenter, D.O., Hong, C.S. and Kannan, K. (2006) PCBs, PCDD/Fs, and organochlorine pesticides in farmed Atlantic salmon from Maine, eastern Canada, and Norway, and wild salmon from Alaska. Environ Sci Technol 40, 5347-54.

Sheehan, D.M. (2000) Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: initial results confirmed. Proc Soc Exp Biol Med 224, 57-60.

Shekhar, P.V., Werdell, J. and Basrur, V.S. (1997) Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J Natl Cancer Inst 89, 1774-82.

Shimizu, K., Ogawa, F., Thiele, J.J., Bae, S. and Sato, S. (2007) Lipid peroxidation is enhanced in Yusho victims 35 years after accidental poisoning with polychlorinated biphenyls in Nagasaki, Japan. J Appl Toxicol 27, 195-7.

Shimokawa, N., Miyazaki, W., Iwasaki, T. and Koibuchi, N. (2006) Low dose hydroxylated PCB induces c-Jun expression in PC12 cells. Neurotoxicology 27, 176-83.

Shipp, E.B., Restum, J.C., Bursian, S.J., Aulerich, R.J. and Helferich, W.G. (1998) Multigenerational study of the effects of consumption of PCB-contaminated carp from Saginaw Bay, Lake Huron, on mink. 3. Estrogen receptor and progesterone receptor concentrations, and potential correlation with dietary PCB consumption. J Toxicol Environ Health A 54, 403-20.

Silkworth, J.B., Antrim, L. and Kaminsky, L.S. (1984) Correlations between polychlorinated biphenyl immunotoxicity, the aromatic hydrocarbon locus, and liver microsomal enzyme induction in C57BL/6 and DBA/2 mice. Toxicol Appl Pharmacol 75, 156-65.

Simmons, S.L., Cummings, J.A., Clemens, L.G. and Nunez, A.A. (2005) Exposure to PCB 77 affects the maternal behavior of rats. Physiol Behav 84, 81-6.

Slim, R., Toborek, M., Robertson, L.W. and Hennig, B. (1999) Antioxidant protection against PCB-mediated endothelial cell activation. Toxicol Sci 52, 232-9.

Smart, J.L., Stephens, D.N. and Katz, H.B. (1983) Growth and development of rats artificially reared on a high or a low plane of nutrition. Br J Nutr 49, 497-506.

Smialowicz, R.J., Andrews, J.E., Riddle, M.M., Rogers, R.R., Luebke, R.W. and Copeland, C.B. (1989) Evaluation of the immunotoxicity of low level PCB exposure in the rat. Toxicology 56, 197-211.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N. and Serrano, F.O. (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103 Suppl 7, 113-22.

Spano, M., Toft, G., Hagmar, L., Eleuteri, P., Rescia, M., Rignell-Hydbom, A., Tyrkiel, E., Zvyezday, V. and Bonde, J.P. (2005) Exposure to PCB and p, p'-DDE in European and Inuit populations: impact on human sperm chromatin integrity. Hum Reprod 20, 3488-99.

Steenland, K., Hein, M.J., Cassinelli, R.T., 2nd, Prince, M.M., Nilsen, N.B., Whelan, E.A., Waters, M.A., Ruder, A.M. and Schnorr, T.M. (2006) Polychlorinated biphenyls and neurodegenerative disease mortality in an occupational cohort. Epidemiology 17, 8-13.

Stehr-Green, P.A., Welty, E., Steele, G. and Steinberg, K. (1986) Evaluation of potential health effects associated with serum polychlorinated biphenyl levels. Environ Health Perspect 70, 255-9.

Steinberg, R., Juenger, T. and Gore, A. (2007) The effects of prenatal PCBs on adult female paced mating reproductive behaviors in rats. Hormones and Behavior article in press.

Steinberg, R.M., Walker, D.M. and Gore, A.C. (Year) Fetal PCB exposure disrupts reproductive physiology and behavior in adulthood. Endocrine Society Forum on Endocrine Disrupting Chemicals,San Diego, CA

Stellman, S.D., Djordjevic, M.V., Muscat, J.E., Gong, L., Bernstein, D., Citron, M.L., White, A., Kemeny, M., Busch, E. and Nafziger, A.N. (1998) Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. Cancer Epidemiol Biomarkers Prev 7, 489-96.

Storey, J.D. and Tibshirani, R. (2003) Statistical methods for identifying differentially expressed genes in DNA microarrays. Methods Mol Biol 224, 149-57.

Sunahara, G.I., Nelson, K.G., Wong, T.K. and Lucier, G.W. (1987) Decreased human birth weights after in utero exposure to PCBs and PCDFs are associated with decreased placental EGF-stimulated receptor autophosphorylation capacity. Mol Pharmacol 32, 572-8.

Szyf, M., Weaver, I.C., Champagne, F.A., Diorio, J. and Meaney, M.J. (2005) Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. Front Neuroendocrinol 26, 139-62.

Takagi, Y., Aburada, S., Hashimoto, K. and Kitaura, T. (1986) Transfer and distribution of accumulated (14C)polychlorinated biphenyls from maternal to fetal and suckling rats. Arch Environ Contam Toxicol 15, 709-15.

Takser, L., Mergler, D., Baldwin, M., De Grosbois, S., Smargiassi, A. and Lafond, J. (2005) Thyroid hormones in pregnancy in relation to environmental exposure to organochlorine compounds and mercury. Environ Health Perspect 113, 1039-45.

Tamas, A., Lubics, A., Lengvari, I. and Reglodi, D. (2006) Effects of age, gender, and gonadectomy on neurochemistry and behavior in animal models of Parkinson's disease. Endocrine 29, 275-87.

Tan, Y., Li, D., Song, R., Lawrence, D. and Carpenter, D.O. (2003) Ortho-substituted PCBs kill thymocytes. Toxicol Sci 76, 328-37.

Tan, Y., Chen, C.H., Lawrence, D. and Carpenter, D.O. (2004a) Ortho-substituted PCBs kill cells by altering membrane structure. Toxicol Sci 80, 54-9.

Tan, Y., Song, R., Lawrence, D. and Carpenter, D.O. (2004b) Ortho-substituted but not coplanar PCBs rapidly kill cerebellar granule cells. Toxicol Sci 79, 147-56.

Tanabe, S., Nakagawa, Y. and Tatsukawa, R. (1981) Aborption Efficiency and Bio.ogical Half-Life of Individual Chlorobiphenyls in Rats Treated with Kanechlor Products. Agric Biol Chem 45, 717-26.

Tavolari, S., Bucci, L., Tomasi, V. and Guarnieri, T. (2006) Selected polychlorobiphenyls congeners bind to estrogen receptor alpha in human umbilical vascular endothelial (HUVE) cells modulating angiogenesis. Toxicology 218, 67-74.

Taylor, P.R., Lawrence, C.E., Hwang, H.L. and Paulson, A.S. (1984) Polychlorinated biphenyls - Influence on birth weight and gestation. Am J Publ Health 74, 1153-4.

Thomas, G., Sweetman, A., Ockenden, W., Mackay, D. and Jones, K. (1998a) Air-Pasture Transfer of PCBs. Environmental Science and Technology 32, 936-42.

Thomas, G., Sweetman, A.J., Ockenden, W.A., Mackay, D. and Jones, K.C. (1998b) Air-Pasture Transfer of PCBs. Environmental Sciences Technology 32, 936-42.

Tiido, T., Rignell-Hydbom, A., Jonsson, B., Giwercman, Y.L., Rylander, L., Hagmar, L. and Giwercman, A. (2005) Exposure to persistent organochlorine pollutants associates with human sperm Y:X chromosome ratio. Hum Reprod 20, 1903-9.

Tiido, T., Rignell-Hydbom, A., Jonsson, B.A., Giwercman, Y.L., Pedersen, H.S., Wojtyniak, B., Ludwicki, J.K., Lesovoy, V., Zvyezday, V., Spano, M., Manicardi, G.C., Bizzaro, D., Bonefeld-Jorgensen, E.C., Toft, G., Bonde, J.P., Rylander, L., Hagmar, L. and Giwercman, A. (2006) Impact of PCB and p,p'-DDE contaminants on human sperm Y:X chromosome ratio: studies in three European populations and the Inuit population in Greenland. Environ Health Perspect 114, 718-24.

Tilson, H.A., Kodavanti, P.R., Mundy, W.R. and Bushnell, P.J. (1998) Neurotoxicity of environmental chemicals and their mechanism of action. Toxicol Lett 102-103, 631-5.

Tobet, S.A. and Fox, T.O. (1989) Sex- and hormone-dependent antigen immunoreactivity in developing rat hypothalamus. Proc Natl Acad Sci U S A 86, 382-6.

Toborek, M., Barger, S.W., Mattson, M.P., Espandiari, P., Robertson, L.W. and Hennig, B. (1995) Exposure to polychlorinated biphenyls causes endothelial cell dysfunction. J Biochem Toxicol 10, 219-26.

Troster, A.I., Ruff, R.M. and Watson, D.P. (1991) Dementia as a neuropsychological consequence of chronic occupational exposure to polychlorinated biphenyls (PCBs). Arch Clin Neuropsychol 6, 301-18.

Tryphonas, L., Truelove, J., Zawidzka, Z., Wong, J., Mes, J., Charbonneau, S., Grant, D.L. and Campbell, J.S. (1984) Polychlorinated biphenyl (PCB) toxicity in adult cynomolgus monkeys (M. fascicularis): a pilot study. Toxicol Pathol 12, 10-25.

Tryphonas, L., Arnold, D.L., Zawidzka, Z., Mes, J., Charbonneau, S. and Wong, J. (1986) A pilot study in adult rhesus monkeys (M. mulatta) treated with Aroclor 1254 for two years. Toxicol Pathol 14, 1-10.

Tsai, M.L., Webb, R.C. and Loch-Caruso, R. (1997) Increase of oxytocin-induced oscillatory contractions by 4-hydroxy-2',4',6'-trichlorobiphenyl is estrogen receptor mediated. Biol Reprod 56, 341-7.

Tsai, P.C., Ko, Y.C., Huang, W., Liu, H.S. and Guo, Y.L. (2007) Increased liver and lupus mortalities in 24-year follow-up of the Taiwanese people highly exposed to polychlorinated biphenyls and dibenzofurans. Sci Total Environ 374, 216-22.

Turk, D.E. and Hietman, K.G. (1976) Ingested polychlorinated biphenyl Aroclor 1242 and growth, calcium and zinc absorption, and intestinal ultrastructure in chicks. Poult Sci 55, 672-8.

Turusov, V.S., Trukhanova, L.S., Parfenov Yu, D. and Tomatis, L. (1992) Occurrence of tumours in the descendants of CBA male mice prenatally treated with diethylstilbestrol. Int J Cancer 50, 131-5.

Twaroski, T.P., O'brien, M.L., Larmonier, N., Glauert, H.P. and Robertson, L.W. (2001) Polychlorinated biphenyl-induced effects on metabolic enzymes, AP-1 binding, vitamin E, and oxidative stress in the rat liver. Toxicol Appl Pharmacol 171, 85-93.

Uphouse, L. and Caldarola-Pastuszka, M. (1993) Female sexual behavior following intracerebral infusion of the 5-HT1A agonist, 8-OH-DPAT, into the medial preoptic area. Brain Res 601, 203-8.

Valdez, G.R., Sabino, V. and Koob, G.F. (2004) Increased anxiety-like behavior and ethanol self-administration in dependent rats: reversal via corticotropin-releasing factor-2 receptor activation. Alcohol Clin Exp Res 28, 865-72.

Venkataraman, P., Muthuvel, R., Krishnamoorthy, G., Arunkumar, A., Sridhar, M., Srinivasan, N., Balasubramanian, K., Aruldhas, M.M. and Arunakaran, J. (2006) PCB

(Aroclor 1254) enhances oxidative damage in rat brain regions: Protective role of ascorbic acid. Neurotoxicology
Verreault, J., Bech, C., Letcher, R.J., Ropstad, E., Dahl, E. and Gabrielsen, G.W. (2007) Organohalogen contamination in breeding glaucous gulls from the Norwegian Arctic: associations with basal metabolism and circulating thyroid hormones. Environ Pollut 145, 138-45.

Vettori, M.V., Goldoni, M., Caglieri, A., Poli, D., Folesani, G., Ceccatelli, S. and Mutti, A. (2006) Antagonistic effects of methyl-mercury and PCB153 on PC12 cells after a combined and simultaneous exposure. Food Chem Toxicol 44, 1505-12.

Viau, V. and Meaney, M.J. (1996) The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. J Neurosci 16, 1866-76.

Viau, V. (2002) Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. J Neuroendocrinol 14, 506-13.

Vivian, J.A. and Miczek, K.A. (1993) Diazepam and gepirone selectively attenuate either 20-32 or 32-64 kHz ultrasonic vocalizations during aggressive encounters. Psychopharmacology (Berl) 112, 66-73.

Voie, O.A. and Fonnum, F. (2000) Effect of polychlorinated biphenyls on production of reactive oxygen species (ROS) in rat synaptosomes. Arch Toxicol 73, 588-93.

Vom Saal, F.S. (1989) Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. J Anim Sci 67, 1824-40.

Vomachka, M.S., Vodicnik, M.J. and Lech, J.J. (1983) Characteristics of 2,4,5,2',4',5'-hexachlorobiphenyl distribution among lipoproteins in vitro. Toxicol Appl Pharmacol 70, 350-61.

Wagner, C.K., Nakayama, A.Y. and De Vries, G.J. (1998) Potential role of maternal progesterone in the sexual differentiation of the brain. Endocrinology 139, 3658-61.

Wakui, S., Yokoo, K., Takahashi, H., Muto, T., Suzuki, Y., Kanai, Y., Hano, H., Furusato, M. and Endou, H. (2006) Prenatal 3,3',4,4',5-pentachlorobiphenyl exposure modulates induction of rat hepatic CYP 1A1, 1B1, and AhR by 7,12-dimethylbenz[a]anthracene. Toxicol Appl Pharmacol 210, 200-11.

Walker, B.E. and Haven, M.I. (1997) Intensity of multigenerational carcinogenesis from diethylstilbestrol in mice. Carcinogenesis 18, 791-3.

Wallace, V.C., Norbury, T.A. and Rice, A.S. (2005) Ultrasound vocalisation by rodents does not correlate with behavioural measures of persistent pain. Eur J Pain 9, 445-52.

Wang, S.L., Chen, T.T., Hsu, J.F., Hsu, C.C., Chang, L.W., Ryan, J.J., Guo, Y.L. and Lambert, G.H. (2003) Neonatal and childhood teeth in relation to perinatal exposure

to polychlorinated biphenyls and dibenzofurans: observations of the Yucheng children in Taiwan. Environ Res 93, 131-7.

Wang, X.Q., Fang, J., Nunez, A.A. and Clemens, L.G. (2002) Developmental exposure to polychlorinated biphenyls affects sexual behavior of rats. Physiol Behav 75, 689-96.

Wassermann, D., Wassermann, M., Cucos, S. and Djavaherian, M. (1973) Function of adrenal gland-zona fasciculata in rats receiving polychlorinated biphenyls. Environ Res 6, 334-8.

Weaver, I.C., Cervoni, N., Champagne, F.A., D'alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M. and Meaney, M.J. (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7, 847-54.

Weber, R. and Sakurai, T. (2001) Formation characteristics of PCDD and PCDF during pyrolysis processes. Chemosphere 45, 1111-7.

Weisskopf, M.G., Anderson, H.A. and Hanrahan, L.P. (2003) Decreased sex ratio following maternal exposure to polychlorinated biphenyls from contaminated Great Lakes sport-caught fish: a retrospective cohort study. Environ Health 2, 2.

Weltje, L., Vom Saal, F.S. and Oehlmann, J. (2005) Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. Hum Exp Toxicol 24, 431-7.

Wersinger, S.R., Baum, M.J. and Erskine, M.S. (1993) Mating-induced FOS-like immunoreactivity in the rat forebrain: a sex comparison and a dimorphic effect of pelvic nerve transection. J Neuroendocrinol 5, 557-68.

Wheaton, J.E., Krulich, L. and Mccann, S.M. (1975) Localization of luteinizing hormone-releasing hormone in the preoptic area and hypothalamus of the rat using radioimmunoassay. Endocrinology 97, 30-8.

Wojtowicz, A.K., Goch, M. and Gregoraszczuk, E.L. (2005) Polychlorinated biphenyls (PCB 153 and PCB 126) action on conversion of 20-hydroxylated cholesterol to progesterone, androstenedione to testosterone, and testosterone to estradiol 17beta. Exp Clin Endocrinol Diabetes 113, 464-70.

Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C. and Paules, R.S. (2001) Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol 8, 625-37.

Woodhouse, A.J. and Cooke, G.M. (2004) Suppression of aromatase activity in vitro by PCBs 28 and 105 and Aroclor 1221. Toxicol Lett 152, 91-100.

Wrobel, M. and Kotwica, J. (2005) Effect of polychlorinated biphenyls (PCBs) on basal and OT-stimulated calcium concentrations in myometrial cells in cows. Reprod Biol 5, 321-30.

Xu, J., Stouffer, R.L., Searles, R.P. and Hennebold, J.D. (2005) Discovery of LH-regulated genes in the primate corpus luteum. Mol Hum Reprod 11, 151-9.

Xu, J., Hennebold, J.D. and Stouffer, R.L. (2006) Dynamic expression and regulation of the corticotropin-releasing hormone/urocortin-receptor-binding protein system in the primate ovary during the menstrual cycle. J Clin Endocrinol Metab 91, 1544-53.

Yajima, Y., Hayashi, Y. and Yoshii, N. (1981) Identification of ultrasonic vocalization substrates determined by electrical stimulation applied to the medulla oblongata in the rat. Brain Res 229, 353-62.

Yamane, T., Fukuda, N., Inaba, J. and Nishimura, Y. (1975) Effect of polychlorinated biphenyls (PCB) on metabolism of thyroid hormone in Wistar rats. Nippon Eiseigaku Zasshi 30, 497-502.

Yamashita, F. and Hayashi, M. (1985) Fetal PCB syndrome: clinical features, intrauterine growth retardation and possible alteration in calcium metabolism. Environ Health Perspect 59, 41-5.

Yang, C.Y., Yu, M.L., Guo, H.R., Lai, T.J., Hsu, C.C., Lambert, G. and Guo, Y.L. (2005) The endocrine and reproductive function of the female Yucheng adolescents prenatally exposed to PCBs/PCDFs. Chemosphere 61, 355-60.

Yang, J.H. and Kodavanti, P.R. (2001) Possible molecular targets of halogenated aromatic hydrocarbons in neuronal cells. Biochem Biophys Res Commun 280, 1372-7.

Yen, S.S. and Lein, A. (1976) The apparent paradox of the negative and positive feedback control system on gonadotropin secretion. Am J Obstet Gynecol 126, 942-54.

Yoon, K., Pallaroni, L., Stoner, M., Gaido, K. and Safe, S. (2001) Differential activation of wild-type and variant forms of estrogen receptor alpha by synthetic and natural estrogenic compounds using a promoter containing three estrogen-responsive elements. J Steroid Biochem Mol Biol 78, 25-32.

Yu, M.L., Hsu, C.C., Guo, Y.L., Lai, T.J., Chen, S.J. and Luo, J.M. (1994) Disordered behavior in the early-born Taiwan Yucheng children. Chemosphere 29, 2413-22.

Yu, M.L., Hsin, J.W., Hsu, C.C., Chan, W.C. and Guo, Y.L. (1998) The immunologic evaluation of the Yucheng children. Chemosphere 37, 1855-65.

Yu, M.L., Guo, Y.L., Hsu, C.C. and Rogan, W.J. (2000) Menstruation and reproduction in women with polychlorinated biphenyl (PCB) poisoning: long-term follow-up interviews of the women from the Taiwan Yucheng cohort. Int J Epidemiol 29, 672-7.

Zhang, Y., Wise, J.P., Holford, T.R., Xie, H., Boyle, P., Zahm, S.H., Rusiecki, J., Zou, K., Zhang, B., Zhu, Y., Owens, P.H. and Zheng, T. (2004) Serum polychlorinated biphenyls, cytochrome P-450 1A1 polymorphisms, and risk of breast cancer in Connecticut women. Am J Epidemiol 160, 1177-83.

Zoeller, R.T., Dowling, A.L. and Vas, A.A. (2000) Developmental exposure to polychlorinated biphenyls exerts thyroid hormone-like effects on the expression of RC3/neurogranin and myelin basic protein messenger ribonucleic acids in the developing rat brain. Endocrinology 141, 181-9.

Vita

Rebecca Meg Steinberg was born on February 20th, 1977 in Ravenna, Ohio. She has one older brother and one younger sister, neither of who pursued a career in science. Rebecca spent her senior year of high school studying abroad in France and then matriculated at Barnard College in the year 1995, and transferred to the University of Chicago in 1996. From 1997-1998 she worked in Dublin, Ireland, and London, England in the fields of international hostel management and scientific publishing. In 2000, Rebecca graduated from the University of Chicago with a joint BA in Neuroscience and Ecology & Evolution, and then entered the Institute for Neuroscience PhD program at the University of Texas in Austin. From the years 2000-2003, Rebecca worked with Dr. George Pollak in the field of bat vocalization neuroethology, but her research interests in neuroendocrinology led her to the laboratory of Dr. Andrea Gore. The dissertation research presented in this publication results from her work with Dr. Gore from 2003-2006 on the toxicological effects of prenatal PCB exposure on adult female reproduction, across development, behavior, and gene expression.

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